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RETENTION OF THE NITROGEN OF AMINO ACIDS ADMINISTERED SINGLY OR IN MIXTURES TO DOGS FED DIETS LOW IN PROTEIN[1]

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Little is known concerning the functions and fates of the amino acids required for maintenance. Mitchell and Hamilton have pointed out that, although for the building up of the protein molecule, the requirements for the various amino acids are presumably interdependent in nature, the requirement for an amino acid for maintenance, for meeting certain needs, may not be limited directly by the supply of any other amino acid. Evidence concerning this latter possibility might be obtained by studying what happens to the nitrogen of a needed amino acid administered to an adult animal supplied with a diet furnishing all requirements except those for nitrogen. If the amino acid can not be used alone, catabolism should be prompt; if it can be used alone, a decrease in the negative balance for nitrogen may be observed. Such an effect, for convenience of discussion, will be attributed to retention of nitrogen, although of course it may result from a sparing of tissue nitrogen.

A number of years ago (1933) we reported in a preliminary communication, the results of experiments to determine possible differences in treatment accorded to a number of amino acids administered singly to animals starving for nitrogen. The conclusion was expressed that there was a distinct retention of a portion of the nitrogen of certain amino acids, but not of that of others. With an increased number of observations of this nature, we still have the impression that the nitrogen of some amino acids is more likely to be retained and indeed to be retained to a greater extent

[1] Based on theses submitted to the Faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy by Louis P. Gerber, June 1933 and by Ernest K. Nielsen, June 1937.

than that of others. However as some of the nitrogen of each amino acid studied has been retained on occasion, conclusive evidence of such a difference would require statistical treatment of a larger number of satisfactory experiments than it would appear profitable to perform at present.

While the amounts of nitrogen retained have been small and not constant when amino acids have been administered singly, it seemed reasonable to anticipate that the nitrogen retained from a number of sources might be cumulated, and by its amount, furnish unmistakable evidence of selective retention. Seuffert and Marks (1925) have found that the nitrogen lost daily by a dog on a protein-free diet was reduced from 3.0 to 1.7 grams following the addition to the ration of asparagine, leucine, tyrosine, tryptophane, glutamic acid and alanine.

While considering the selection of mixtures to be used, we recalled the reports of Abderhalden that he had been able to keep a dog (1912) and rats and mice (1922) in nitrogen balance, for short periods, with diets supplying nitrogen by mixtures of amino acids. The period during which nitrogen equilibrium continued in the dog was only eight days, and he presented no complete data for the experiments with the rats and mice, but it seemed of interest to check his observations. His mixtures contained amino acids then (1922) recognized as essential, and others since shown to be essential in the growing animal. It was decided to use a mixture of these, cystine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophane and valine. As methionine had been shown by Jackson and Block (1932) to replace cystine, there have been employed three other mixtures, like this except that one lacked cystine, one had cystine replaced by methionine and one contained methionine as well as cystine.

EXPERIMENTAL. Short-haired adult female dogs in good nutritive condition, free of intestinal worms, have been fed constant daily amounts of one of the basal rations until the values for fecal and urinary nitrogen have become reasonably constant. The dose of each compound to be studied together with a little of the basal ration for that day has been administered on the morning of the first day of a two-day period. At times a second dose has been given the second day. Two two-day control periods routinely intervened before any other compound was studied.

Three satisfactory experiments each have been performed with lysine, phenylalanine and urea, four each with alanine, glycine and tyrosine, five with arginine, six with cystine, eight with glutamic acid and nine with histidine. Contamination of the urine with fecal material, or regurgitation during the period the compound was administered, or during the period following has made a number of additional experiments less than completely satisfactory. In many such cases the changes in the nitrogen balances have been of the same general significance as have those of the other experiments. Table 1 records figures for representative experiments

TABLE 1

Disposal of the nitrogen of amino acids administered to the dog fed a protein-free diet

PERIOD*	URINE		FECAL N	
	Urea and ammonia N	Total N		
	gm.	*gm.*	*gm.*	
6		1.43	0.46	Dog 3. 7.7 kilo. 130 gm. Ration 3 daily
7		2.64	0.34	1.6 gm. N as creatine, first day
8		1.42	0.38	
9		1.39	0.30	
10		2.29	0.44	0.80 gm. N as glycine, first day
11		1.53	0.46	7.7 kilo
4		1.35	0.50	8.0 kilo
5		1.90	0.36	0.72 gm. N as *l*(−)-tyrosine, first day
6		1.36	0.50	
7		1.38	0.52	
8		1.19	0.48	0.69 gm. N as *l*(−)-cystine, first day
9		0.97	0.50	8.0 kilo
5		2.97	0.24	Dog 6. 8.6 kilo. 100 gm. Ration 3 daily
6		3.64	0.28	0.9 gm. N as *l*(−)-histidine, first day
7		2.64	0.28	
8		2.36	0.26	
9		2.91	0.38	0.9 gm. N as *l*(−)-leucine, first day
10		1.86	0.32	
11		2.23	0.34	
12		2.71	0.36	0.8 gm. N as *dl*-phenylalanine, first day
13		1.86	0.28	8.3 kilo
4a	1.36	2.34	0.28	Dog 6. 9.3 kilo. 130 gm. Ration 3 daily
5a	1.23	2.27	0.34	0.45 gm. N as *l*(−)-histidine, first day
6a	1.10	1.80	0.24	9.3 kilo
5	1.31	1.92	0.30	Dog 8. 7.2 kilo. 110 gm. Ration 3 daily
6	1.41	1.90	0.30	
7	1.71	2.37	0.42	0.7 gm. N as *dl*-alanine, first day
8	1.14	1.62	0.42	
9	1.00	1.48	0.60	
10	1.45	2.04	0.48	0.7 gm. N as *dl*-alanine, first day
11	0.74	1.17	0.46	
12	0.63	1.12	0.38	
13	1.32	1.92	0.52	0.7 gm. N as *l*(+)-arginine, first day
14	0.72	1.16	0.40	
15	0.65	1.13	0.54	

* Periods numbered from beginning of protein-free regimen. Two days per period.

TABLE 1—*Concluded*

PERIOD*	URINE			
	Urea and ammonia N	Total N	Fecal N	
	gm.	*gm.*	*gm.*	
16	1.09	1.56	0.40	0.7 gm. N as *l*(+)-glutamic acid, first day
17	0.62	1.10	0.52	
18	0.52	0.98	0.44	
19	0.72	1.42	0.44	0.7 gm. N as *l*(+)-arginine, first day
20	0.45	0.96	0.42	
21	0.39	0.85	0.52	
22	0.71	1.25	0.44	0.42 gm. N as *l*(−)-phenylalanine, first day
23	0.38	0.84	0.48	7.1 kilo
6	1.08	1.72	0.48	Dog 9. 8.0 kilo. 110 gm. Ration 4 daily
7	1.00	1.61	0.58	0.40 gm. N as *l*(+)-lysine in 2 daily doses
8	0.95	1.58	0.50	of 0.20 gm. N
15	1.05	1.60	0.58	
16	1.24	1.83	0.54	0.40 gm. N as *l*(+)-arginine in 2 daily
17	0.97	1.51	0.44	doses of 0.20 gm. N
18	1.11	1.65	0.52	
19	1.65	2.22	0.44	0.75 gm. N as urea, first day
20	1.03	1.56	0.36	7.0 kilo

with glycine, tyrosine, leucine, lysine, glutamic acid, alanine and urea respectively and for an experiment with creatine. To indicate the range of results obtained, there are included figures for two experiments each for histidine and phenylalanine and for three experiments with arginine. To conserve space, the daily urinary values of the separate urines for each two-day period have been added together for tabulation. This leads to no difficulty of interpretation but it is to be emphasized that changes in the level of urinary nitrogen have been followed more closely than the recorded figures appear to indicate.

The general procedure in the experiments with the mixtures of amino acids has been the same as for those with individual compounds. As the results with the two dogs used were in essential agreement, those for one dog only are presented (table 2). The fecal nitrogen was determined for two-day periods, but is recorded as the average daily excretion.

Daily urinary samples have been separated by catheterization; fecal samples for two-day periods, by the aid of carmine markers. Total nitrogen of the urine, of the feces and of the food has been determined by the Kjeldahl technic; urinary urea and ammonia nitrogen, by the method of Van Slyke and Cullen; urinary creatinine, by Folin's colorimetric pro-

cedure. The compounds administered have been prepared in this laboratory or obtained from Hoffman-La Roche, Inc., or the Eastman Kodak Company. Analysis for total nitrogen or for amino nitrogen has indicated

TABLE 2

Retention of nitrogen of mixtures of amino acids administered to a dog fed a diet low in protein

DAY	URINE			
	Urea and ammonia N	Total N	Fecal N	
	gm.	*gm.*	*gm.*	
18	0.33	0.61	0.17	Dog 9. 7.8 kilo. 130 gm. Ration 6 daily
19	0.32	0.60	0.17	containing 0.25 gm. nitrogen
20	0.31	0.60	0.17	
21	0.31	0.60	0.17	
22	0.31	0.61	0.10	0.24 gm. N as amino acid
23	0.18	0.49	0.10	Mixture *a*, added to ration of day 22
24	0.26	0.54	0.21	
25	0.32	0.55	0.21	
26	0.26	0.55	0.21	0.27 gm. N as amino acid
27	0.18	0.46	0.21	Mixture *b*, added to ration of day 26
28	0.18	0.45	0.18	
29	0.20	0.48	0.18	
30	0.21	0.51	0.18	
31	0.21	0.48	0.18	
32	0.23	0.50	0.11	0.24 gm. N as amino acid
33	0.18	0.45	0.11	Mixture *c*, added to ration of day 32
34	0.19	0.47	0.20	
35	0.19	0.48	0.20	
36	0.24	0.53	0.19	0.21 gm. N as amino acid
37	0.24	0.52	0.19	Mixture *d*, added to ration of day 36
38	0.19	0.50	0.16	
39	0.20	0.53	0.16	
40	0.25	0.55	0.17	
41	0.23	0.53	0.17	0.21 gm. N as amino acid
42	0.19	0.49	0.17	Mixture *d*, added to ration of day 41 7.8 kilo

Mixture *a*—cystine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophane and valine; 30 mgm. of N provided by each.

Mixture *b*—Same as *a* except 30 mgm. of N as methionine added.

Mixture *c*—Same as *a* except N of cystine replaced by that of methionine.

Mixture *d*—Same as *a* except cystine omitted.

the compounds were satisfactory for use. Diet 3, containing 0.01–0.02 per cent nitrogen, had the following composition: dextrin, 30.1; sucrose, 25.2; lard, 36.0; codliver oil, 3.6; bone ash, 2.9; and Cowgill's salt mixture

2.2 per cent. Diet 4, containing the same amount of nitrogen, differed in that butter fat replaced the lard. These diets have been supplemented daily with 1 gram of dried brewer's yeast. Diet 6, containing 0.19 per cent nitrogen, had the following composition: sucrose, 68.0; vitavose, 9.4; lard, 13.6; butter fat, 5.7; bone ash, 2.2; and Cowgill's salt mixture, 1.1 per cent. The daily allotment of food for each dog has been divided into two or three portions for feeding at intervals through the day. The animals as a rule ate all or the bulk of the food readily. Any remainder was administered by hand.

Discussion of results. The constancy of the excretion of creatinine, except after administration of creatine, has been taken to evidence satisfactory collection of the daily urinary samples. The figures for creatinine are not recorded but it may be noted that they furnish no evidence that under the conditions employed here, the administration of the amino acids studied affects significantly the excretion of creatinine. That the bulk of each of the administered compounds has been absorbed is indicated by the figures for fecal nitrogen. Changes in the urinary nitrogen may therefore be taken to furnish information concerning the proximate fates of the compounds.

In general it is to be observed (table 1) that while there has been some retention, the bulk of the nitrogen of most of the compounds administered has appeared promptly in the urine and furthermore that catabolism has occurred since, when they were determined, urea and ammonia accounted for nearly all of the extra nitrogen. The period following the administration, and indeed usually the day following the administration, urinary values returned to the control levels.

At times, as with glycine and with one experiment with arginine, extra nitrogen appearing in the urine exceeded that fed. Stimulation of catabolism of tissue protein may account for this. It has been found in several experiments that apparently all the nitrogen of cystine has been retained and that the excretion of nitrogen has been reduced during and after the experimental period. While the reported damage of cystine to the kidney (cf. Cox et al., 1929) came to mind as an explanation, there has been observed no albuminuria nor reduced urinary volume to support such a view.

While about three-fourths of the nitrogen of a large dose of histidine was rejected (dog six, period six) all the nitrogen of a small dose was found to be retained (dog 6, period 5a). This result with histidine, and the result with small doses of lysine on successive days (dog 9) may be contrasted with the findings with small daily doses of arginine (dog 9). The decrease in urinary nitrogen following the administration of lysine, and histidine and also cystine, suggest that perhaps the amounts retained exerted a sparing action on body protein continuing for several days.

While most of it has been excreted at once, a portion of the nitrogen of

creatine (cf. Benedict and Osterberg) and of urea (cf. Kocher and Torbert) respectively has been retained.

Mezincesco (1931) has reported that glycine, alanine, aspartic acid and glutamic acid respectively exert a definite sparing effect on body nitrogen. Terroine, Mezincesco and Valla (1933) observed a similar effect of cystine. Stekol (1934) has found that the bulk of the sulfur of l(−)cystine is retained by the adult dog whereas that of d(+)cystine is not. While absorption in these experiments was apparently not checked by fecal analysis, our observations indicate ready absorption of the l(−)cystine under similar experimental conditions. Stekol has also reported the retention of the sulfur of both isomers of methionine by the adult dog maintained on a diet low in protein.

The nitrogen of the mixtures of amino acids used has been absorbed completely. The absence of any significant rise in urinary values indicated complete retention of the nitrogen of mixtures of *a*, *b*, and *c* and of mixture *d* in one of the two experiments. It is of interest that the excretion dropped on subsequent days as though there were a continuing sparing effect on tissue nitrogen of nitrogen that had been retained. Maksimova has reported that the addition of a mixture of tryptophane, histidine, cystine, and tyrosine to the protein-free diet of dogs has resulted in a reduced excretion of nitrogen in the urine, although use of a mixture of alanine, leucine and glutamic acid was followed by an increase in urinary nitrogen. Mixture *d* lacking both cystine and methionine seemed less satisfactory.

While the nitrogen of the mixtures was equivalent to less than half of that catabolized daily, the evidence of selective retention or utilization was so striking that it appeared profitable to study intensively the influence of various mixtures on nitrogen balance. For reasons of economy a smaller animal, the white rat, has been employed for such further investigations. Results of these will be reported later.

SUMMARY

1. A portion of the nitrogen of each of a number of amino acids, of creatine and of urea, administered to dogs fed on diets lacking protein, has on occasion been retained, but with most of the compounds the bulk of the nitrogen has been rejected promptly. Cystine, histidine and lysine have appeared exceptional among the amino acids studied as experiments with them have furnished evidence suggesting selective retention of nitrogen.

2. Mixtures of histidine, isoleucine, leucine, lysine, phenylalanine, tryptophane and valine together with cystine or methionine or both, have been administered to dogs fed on diets low in protein, in amounts to supply nitrogen equal to less than half of the urinary nitrogen. The nitrogen has been retained completely or has spared equivalent amounts of tissue nitrogen.

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RETENTION OF THE NITROGEN OF MIXTURES OF AMINO ACIDS ADMINISTERED TO RATS FED DIETS LOW IN PROTEIN[1]

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The bulk of the nitrogen of selected mixtures of tryptophane, histidine, phenylalanine, isoleucine, leucine, valine, lysine, or of these with cystine, with methionine or with both cystine and methionine, administered to dogs, fed diets low in protein, has been found to be retained or to spare an equivalent amount of nitrogen of the tissues (Nielsen, Gerber and Corley). These experiments led logically to others to learn if these mixtures could bring about nitrogen equilibrium. As the large quantities of amino acids required for extensive investigations with an animal as large as the dog made the cost prohibitive, the rat was selected as the subject for further work. Besides its small size, another advantage of this animal is its inability to regurgitate. Catheterization has made possible metabolism experiments of the accuracy considered necessary. We are reporting here that on a diet low in protein the nitrogen of selected mixtures of amino acids spares markedly the nitrogen of the tissues of the rat as it does that of the dog.

EXPERIMENTAL. The adult female white rats were kept individually in Hendryx circular cages, placed upside down on a removable screen of 16 mesh, resting on a large funnel. The open top of the cage was covered. Feces and occasional lumps of food caught by the screen wire were collected frequently to minimize contamination of the feces by the urine or of the urine by feces or food. Urine draining into a bottle, containing a few drops of toluene, was transferred periodically to a larger vessel kept in the refrigerator. The basal diet was available at all times in a cup, kept in a holder that prevented practically all scattering. The bulk of the ration was kept in the refrigerator and transferred in weighed amounts to the cups in the cages as necessary. The mixture of amino acids for each three-day period was mixed with about 12 ml. of water and stirred to give a

[1] Based on a thesis submitted by Ernest K. Nielsen to the Faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June 1937.

suspension capable of passing readily through a number 11 rubber catheter. A portion, 2–3 ml., of this suspension was drawn for feeding into an attached 5 ml. syringe. The end of the catheter was rinsed with a few drops of water and passed gently into the rat's stomach. After emptying the syringe, the tube was withdrawn and again rinsed. All rinsings were added to the suspension of amino acids. Feeding took place twice daily. The last day, rinsings of the cup, and syringe, were administered in 1 ml. portions at hourly intervals after the final chief feeding. The last rinsing was fed at least eight hours before the termination of the experimental period.

Feces were separated to correspond to the three-day experimental periods by means of carmine markers. Frequent collection of the pellets simplified the separation. Catheterization completed the collection of the urine for the periods. Interest that has been manifested in our procedure for catheterization of rats warrants a brief description of the technic as applied to adult female rats. With the rat (preferably one that has had young) belly up, a no. 4 French silk catheter lubricated with water or vaseline is inserted nearly parallel to the plane of the body, directed toward the tail. After the catheter has entered about $\frac{1}{4}$ inch, it is brought gently through an angle of about 180°, so that it is pointing towards the head and introduced gently for about an inch. Urine is removed by sucking into an aspirator bottle. If the bladder is full, the sphincter may offer unusual resistance until the catheter has passed, and then relax permitting the urine to escape past the catheter. It is recommended that the entire operation be carried out over a suitable pan, to avoid loss.

The basal ration 6, described in the preceding paper, was consumed readily at most times. If the ingestion fell off, a freshly prepared batch was found to restore the appetite. The consistency was such that little escaped the food containers, and that that did was ordinarily caught by the fine mesh screen.

The amino acids were prepared in this laboratory or purchased from Hoffman LaRoche, Inc., Eastman Kodak Company, Pfanstiehl Chemical Company, or Paul-Lewis Laboratories. Satisfactory purity was established by analysis for total or for amino nitrogen.

Urinary nitrogen, fecal nitrogen and food nitrogen were determined by macro Kjeldahl methods using a digestion mixture containing selenium oxychloride; urinary creatinine was determined by Folin's colorimetric procedure; urinary urea and ammonia nitrogen were determined by Van Slyke and Cullen's method with aeration and titration.

Fecal samples were ground in a mortar with a little water to give a satisfactory suspension which was strained into a 100 ml. volumetric flask through a screen with a fine mesh. Material not passing the screen was ground with water and strained. This was repeated until all material

except a little hair had been dissolved or suspended. After diluting to volume and shaking, aliquots were taken for analysis.

RESULTS. Eleven rats have been under experiment with observations on nitrogen balance for one to two months. Values for urinary creatinine, omitted to conserve space, have evidenced by their constancy, the completeness and accuracy of the collection of the urinary samples. Analyses for fecal nitrogen have indicated complete absorption of the nitrogen of the administered amino acids, and warrant deductions about the fate of this nitrogen, from changes in the urinary picture. To bring out more clearly changes in the loss of nitrogen from the tissues, calculations for nitrogen balance have been made from the available figures. As nitrogen lost by other channels would be expected to be small and relatively constant, no error of interpretation should result.

TABLE 1

Proportional distribution of nitrogen in mixtures of amino acids

SOURCE OF NITROGEN	MIXTURE							
	1	2	3	4	6	8*	11	14
l(−)-Histidine	1	1	1	1	1	1	1	1
l(+)-Isoleucine	1	1	1	1	1	1	1	1
l(−)-Leucine	1	1	1	1	1	1	1	1
l(+)-Lysine	1	1	1	1	1	0.5	0	1
dl-Methionine	1	1	1	1	1	1	1	1
l(−)-Phenylalanine	1	1	1	1	1	1	1	0
l(−)-Tryptophane	1	1	1	1	1	0.5	0.5	1
l(+)-Valine	1	1	1	1	1	1	1	1
α-Amino-β-hydroxybutyric acids	0	1	2	4	6	4	4	4
Total parts of nitrogen	8	9	10	12	14	11	10.5	11

* Interpretation: Of 11 mgm. nitrogen, in mixture 8, there was supplied 0.5 mgm. from lysine and tryptophane respectively, 4 mgm. from α-amino-β-hydroxybutyric acids and 1 mgm. from each of the other amino acids.

A mixture of histidine, isoleucine, leucine, lysine, dl-methionine, phenylalanine, tryptophane and valine prepared so that each amino acid supplied the same amount of nitrogen, has been administered on 13 occasions. In every instance a large portion of the nitrogen has been retained. Extra urinary nitrogen has appeared as urea and ammonia nitrogen. Table 2 records representative experiments. While small amounts of nitrogen appeared to be retained completely at times, it has not been possible to establish nitrogen balance even with the ingestion of nitrogen in an amount equalling that of the urine (rat 9). It appeared probable that these eight amino acids could not meet the requirements for maintenance.

As Rose and associates (1935) had discovered the amino acid threonine

and shown it to be essential for growth, we were interested to learn if the addition of this amino acid would favor the retention of more of the nitrogen of mixtures of amino acids under our experimental conditions. As a source of threonine we have used the mixture of the isomeric α-amino-β-hydroxybutyric acids prepared by the methods of Carter and co-workers

TABLE 2

Disposal of the nitrogen of mixtures of amino acids administered to rats fed a diet low in nitrogen

PERIOD*	FOOD	URINE		FECES TOTAL NITROGEN	NITROGEN OF FOOD†	NITROGEN BALANCE	
		Urea and ammonia nitrogen	Total nitrogen				
	gm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
4	28	81	132	31	53	−110	Rat 1, 283 grams, ration 6
5	31	78	128	50	57	−121	
6	19	116	171	26	115	−82	80 mgm. nitrogen as mixture 1
7	28	63	110	47	52	−105	
8	28	62	109	43	53	−99	
9	26	57	112	35	49	−98	
10	22	73	121	38	113	−46	72 mgm. nitrogen as mixture 1
11	33	60	119	42	62	−99	257 grams weight
5	26	71	120	41	49	−112	Rat 9, 218 grams, ration 6
6	22	75	128	37	42	−123	
7	23	76	130	33	47	−116	
8	22	62	113	15	41	−87	
9	25	66	119	38	48	−109	
10	17	129	190	26	153	−63	120 mgm. nitrogen as mixture 1
11	16	140	201	26	151	−76	120 mgm. nitrogen as mixture 1
12	21	60	113	39	42	−110	
13	24	50	102	36	36	−102	
14	23	53	101	25	43	−83	
15	21	46	90	31	39	−82	
16	17	46	88	28	32	−84	158 grams weight

* Periods numbered from beginning of protein-low regimen. Three days per period.

† Includes nitrogen of mixtures of amino acids.

(1937) to whom we are indebted for suggestions furnished before publication of their later improved procedure. It has been found that the inclusion of threonine in the mixtures of amino acids administered has increased the retention of nitrogen.

Table 3 presents results with one of the four rats used in this phase of the study. As the amount of the α-amino-β-hydroxybutyric acids has

been increased, the urinary nitrogen has *decreased* until a definite positive balance of nitrogen has occurred (period 13), indicating that the nitrogen of the mixture of nine amino acids has been retained completely or that it has spared an equivalent or even greater amount of tissue nitrogen.

TABLE 3

Sparing of nitrogen of the tissues by the nitrogen of mixtures of amino acids administered to a rat fed a diet low in nitrogen

PERIOD*	BODY WEIGHT	FOOD	URINE		FECES TOTAL NITROGEN	NITROGEN OF FOOD†	NITROGEN BALANCE	
			Urea and ammonia nitrogen	Total nitrogen				
	gm.	*gm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
4	222	15	79	129	41	29	−141	Rat 12, ration 6
5	214	18	81	121	35	33	−123	
6	207	19	65	114	37	36	−115	
7	202	21	66	115	41	40	−116	
8	197	23	65	113	36	43	−106	
9	190	18	94	147	18	107	−58	72 mgm. nitrogen as mixture 2
10	188	17	96	150	26	104	−72	72 mgm. nitrogen as mixture 2
11	186	23	82	139	28	123	−44	80 mgm. nitrogen as mixture 3
12	184	20	55	116	28	134	−10	96 mgm. nitrogen as mixture 4
13	188	22	33	96	28	153	19	112 mgm. nitrogen as mixture 6
14	189	25	28	90	27	160	43	112 mgm. nitrogen as mixture 6
15	189	22	29	94	32	154	28	112 mgm. nitrogen as mixture 6
16	190	23	26	83	26	121	12	77 mgm. nitrogen as mixture 8
17	188	24	29	85	30	121	6	77 mgm. nitrogen as mixture 8
18	189	25	35	94	28	121	−1	73.5 mgm. nitrogen as mixture 11 Lysine absent
19	193	23	56	112	33	117	−28	73.5 mgm. nitrogen as mixture 11 Lysine absent
20	193	24	39	88	29	123	6	77 mgm. nitrogen as mixture 8
21	193	20	42	94	23	115	−2	77 mgm. nitrogen as mixture 14 Phenylalanine absent
22	192	14	58	105	15	103	−17	77 mgm. nitrogen as mixture 14 Phenylalanine absent
23	187	18	33	86	22	111	3	77 mgm. nitrogen as mixture 8
24	192	22	34	85	30	120	5	79 mgm. nitrogen as mixture 8 plus 2 mgm. nitrogen as *d*-valine

* Periods numbered from beginning of protein-low regimen. Three days per period.

† Includes nitrogen of mixtures of amino acids.

As nitrogen balance has not been possible in the absence of threonine, it seemed of interest to observe the effect of the absence of one of the other amino acids. Omission of lysine (periods 18 and 19) or of phenylalanine

(periods 21 and 22) from the mixtures has been followed by a decreased retention of nitrogen. Restoration of the omitted amino acid has been followed by a prompt *decrease* in the urinary nitrogen indicating an increased retention of nitrogen. Similar observations have been made following the omission of valine, isoleucine and leucine respectively.

Discussion. Presumably any amino acids needed and not supplied in the diet will be obtained from the protein of the tissues. If catabolism is the only fate of the amino acids left over from the protein used, the nitrogen of the body will not be spared unless all the required amino acids are provided in the food. The results here presented indicate that much of the continuous catabolism of the tissues occurring during starvation for nitrogen can be prevented by supplying mixtures of amino acids which do not meet all the requirements for maintenance. While a satisfactory explanation of these findings is difficult, it may be pointed out that if residual amino acids were used to reconstitute protein of altered composition or pattern (cf. Maksimova, 1936) results like those here reported would be obtained. The fewer the amino acids needed the more efficiently the "left over" ones might be expected to be conserved, and the more marked would be the sparing of the protein of the tissues.

SUMMARY

A considerable portion of the nitrogen of selected mixtures of amino acids has been retained or has spared an equivalent amount of nitrogen of the tissues, following administration to adult female white rats fed diets low in protein.

All of the nitrogen of a mixture of the amino acids threonine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophane and valine has been found to be retained or to spare an equivalent amount of the nitrogen of the tissues, under the experimental conditions used.

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INDUCTION OF ESTROUS BEHAVIOR IN ANESTROUS CATS WITH THE FOLLICLE-STIMULATING AND LUTEINIZING HORMONES OF THE ANTERIOR PITUITARY GLAND[1]

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Estrus is a highly complex biological phenomenon which manifests itself objectively through the physical and emotional reactions of heat and the characteristic histological appearance of the reproductive tract. The behavior pattern of mating, and, to a less extent, the cornified-cell-content of the vagina serve, therefore, as biological indicators of estrus. The hormones of the endocrine glands are obviously important in the development of the sexual drive in estrus. Stone (1926) has demonstrated that the sexual aggressiveness and copulatory ability of male rats gradually diminish and finally disappear within one to seven months after castration. Allen, Doisy and their collaborators (1924) have reported that a certain percentage of rats and mice will experience normal mating reactions after ovariectomy if given adequate substitution therapy by injections of theelin. In others, however, the full estrous growth reaction of the vagina may be obtained without eliciting any evidence of the mating reaction (Hemmingsen, 1929; Wiesner and Mirskaia, 1930). These data suggest that a greater amount of hormone is necessary to excite the mating reaction than is needed to induce vaginal epithelial changes (Marrian and Parkes, 1930). Similar observations were made by Cole and Miller (1935) who induced estrus in anestrous ewes by the injection of an estrogen alone. They, too, found that estrus could be brought on with greater regularity by using adequately large doses of an estrogen. Witschi and Pfeiffer (1935) have also encountered cornification of the vaginal epithelium in their "constant estrus" female rats without evidence of mating instincts, but they attribute the absence of estrous behavior to a deficient supply of luteinizing hormone of the anterior pituitary gland.

Heat can also be produced in spayed female guinea pigs by the injection of an estrogen alone, but the administration of an estrogen followed by injections of progesterone is more effective (Dempsey, Hertz and Young, 1936; Collins, Bolling, Dempsey and Young, 1938). The estrogen-

[1] Aided by a grant from the Committee for Research in Problems of Sex of the National Research Council.

induced heat differs from the estrogen-progesterone-induced heat in several respects, but the significance of these differences is not yet established (Bolling, Young and Dempsey, 1938).

Estrus has been induced in animals by hormones other than an estrogen and progesterone. Cole and Miller (1933 and 1935) produced ovulation and estrus in anestrous ewes with gonadotropic hormone extracted from pregnant mare serum. The gonadotropic hormone had to be given in two single doses of 100 R.U. each, at 16-day intervals, in order to induce estrus as well as ovulation. The length of a ewe's estrous cycle is about 17 days. A single dose of this hormone brought about ovulation only. McKenzie and Terrill (1937) likewise had no difficulty in producing ovulation in 11 anestrous ewes by giving them single injections of pregnant mare's serum; but estrus occurred in only 1 of these animals. Roux (1936) failed to produce estrus in ewes by injections of a standardized anterior pituitary hormone "Prolan."

Because of the well-recognized pituitary-ovarian relationship it seemed pertinent to inquire into the possibility of arousing estrous behavior in anestrous animals by the administration of a highly purified gonadotropic hormone of the anterior pituitary gland. Such a hormone was made available for these experiments through the generosity of Dr. H. L. Fevold.

Experimental method. The extract used was a highly purified fraction of the follicle-stimulating hormone (FSH) which contained traces of the luteinizing hormone (LH). Six healthy young adult female cats were selected from the routine laboratory supply for this experiment. They had been under observation for a period of several weeks in July, 1938, without being found in heat, and it was concluded that they had entered their usual seasonal period of anestrus. The extract was administered to them subcutaneously in daily doses of varying size. The FSH was given either in solution or in the tannated form (table 1). Every day before its injection each cat was tested for estrous behavior with a well-trained male. As soon as the female showed a well-defined pattern of behavior characteristic of estrus (spontaneous rolling, treading, etc.) she was permitted to mate several times. Her reactions were noted during and after coitus in order to judge whether the complete picture of typically spontaneous estrus had occurred. The animals were killed with ether within $3\frac{1}{2}$ to 5 hours after mating, and their ovaries were preserved for histological study.

Results. Five of the 6 cats came into heat between the seventh and thirteenth day of the experimental period. Three of the 6 cats were in heat after the eighth injection. The 6th cat, which failed to come into estrus by the thirteenth day of the experiment, was autopsied at that time and found to be a surgical castrate. The other 5 cats, who submitted to mating eagerly, showed markedly enlarged ovaries at autopsy and the

ovarian follicles were obviously well developed. Microscopic examination of these follicles revealed a histological picture similar in some respects to that obtained by Foster and Hisaw (1936) and Friedgood and Foster (1938), who induced ovulation in anestrous cats by the subcutaneous and intravenous injections of FSH and LH. As these investigators found, it is ordinarily necessary to give the final dose of FSH and LH intravenously to make certain that ovulation takes place. In the present experiments no attempt was made to induce ovulation, and all of the extract was administered subcutaneously. This precaution notwithstanding, 2 of the cats ovulated, presumably just before or soon after mating. Slight to extensive luteinization of the walls of the follicles occurred without ovula-

TABLE 1

CAT NO.	1938													AUTOPSY, HOURS AFTER MATING	OVARIES
	7/13	7/14	7/15	7/16	7/17	7/18	7/19	7/20	7/21	7/22	7/23	7/24	7/25		
C1	F4	F4	F4	F4	F4	F2	+*							5	Normal estrous follicles
C2	F4	F4	F4	F4	F4	F2	F6	F6	+*					6	Luteinization of unovulated follicles
C3	T6	T6	T16	F6	F6	0	F6	F6	+*					4½	Luteinization of unovulated follicles
C4	T6	T6	T16	F6	F6	0	F6	F6	T25	+*				3½	Three ovulation points, luteinization of unovulated follicles
C5	F4	F4	F4	F4	F4	F2	F6	F6	T25	T25	0	0	+*	5	One ovulation point; luteinization of unovulated follicles
C6	F4	F4	F4	F4	F4	F2	F6	F6	T25	T25	0	T25	—*		

Code: T = tannated FSH, F = FSH in solution, Arabic numerals = dose in rat units, + = in heat, — = not in heat.

* Date of autopsy.

tion in 4 of them, and the nuclei of the retained ova were of the normal vesicular variety. In 1 cat only large estrous follicles were found in the ovaries.

Discussion. The accidental finding of a surgical castrate among the 6 cats chosen for this experiment furnished an unexpected but welcome additional control. The fact that she was the only cat which did not come into heat strengthens the evidence culled from the other experiments and indicates, furthermore, that the pituitary extract induces its estrous effect indirectly through the ovaries. The positive results obtained in the other 5 cats demonstrate that typical estrous behavior can be elicited in anestrous cats by injections of an anterior pituitary extract containing FSH with traces of LH.

Luteinization of the ovarian follicles and the occasional occurrence of ovulation were probably due to the traces of LH known to be present in the solution of FSH. Similar observations were made by Foster and Hisaw (1936) and Friedgood and Foster (1938) as a result of injecting anestrous cats with FSH and LH. How much of this LH effect was due, however, to the cat's own pituitary (as a result of pituitary stimulation by the estrogen secreted from an activated ovary) is a question which can be settled only through further experimentation with hypophysectomized anestrous cats.

Estrous behavior in cats has thus been induced by a purified extract of the anterior hypophysis containing the gonadotropic hormones FSH and LH. That the LH played an important rôle in the development of sexual drive (estrus) is suggested by the experiments of Witschi and Pfeiffer (1935). Until further evidence is available one may only conjecture to what extent the hypophysis is primarily responsible for the awakening of estrous behavior of cats when the mating season arrives spontaneously in the normal course of events.

SUMMARY

Five of 6 young adult anestrous cats came into heat within 7 to 13 days after being injected daily with a highly purified extract of the anterior pituitary gland containing follicle-stimulating hormone (FSH) with traces of the luteinizing hormone (LH). The total dosage used in each instance was 22, 34, 52, 77, 84 and 109 rat units of follicle-stimulating hormone (table 1). The cat which received 109 units for 16 days did not come into heat, and when autopsied was found to be a surgical castrate. The behavior of the estrous cats before, during and after mating was identical in every respect with the behavior of cats which come into heat spontaneously. The ovaries of these cats contained normal estrous follicles. Ovulation had occurred in 2 of the ovaries, and extensive luteinization of follicles without ovulation was also noted.

One may conclude that a highly purified fraction of follicle-stimulating hormone containing traces of luteinizing hormone induces estrous behavior in anestrous cats probably by means of ovarian stimulation.

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THE INFLUENCE OF THYROIDECTOMY ON THE PERIOD OF GESTATION IN THE RABBIT[1]

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Considerable experimental evidence exists indicating a relationship between the thyroid gland and the organs of reproduction. In general, the results obtained in experimental animals by the administration of thyroid substance leads to a disturbance of the oestrous cycle (see Weichert, 1930, 1933), but there is lack of agreement concerning the results after thyroid removal. Hofmeister (1894), Tatum (1913) and Kunde, Carlson and Proude (1929) have reported that thyroidectomy is followed by degenerative changes in the organs of reproduction, while Hammett (1926), Loeser (1932) and Smelzer (1934) reported little influence on the ovaries following thyroid removal.

Since Ukita (1920) reported a striking increase in the length of the gestation period in rabbits as a result of thyroid ablation during pregnancy while Hammett (1922) reported no interference in the fertility of rats, a restudy of the effects of thyroid removal was deemed advisable.

MATERIAL AND METHODS. A total of 67 New Zealand does ranging in age from 7 to 15 months were used in this study; 15 served as controls and the remaining 52 were mated and then thyroidectomized at intervals of from 1½ hours to 12 days post coitum. No attempt was made to preserve the parathyroids. The length of the period of gestation was observed and recorded for every animal.

The rate of oxygen consumption was calculated for each animal with a modified Benedict apparatus by averaging three determinations after a starvation period of 18 to 24 hours.

At the completion of the experiments the animals were sacrificed three to six weeks after delivery and the entire larynx and upper part of the trachea removed and fixed in Bouin's fluid. These were carefully examined for traces of thyroid tissue under a dissecting binocular microscope, suspicious pieces being removed, sectioned and stained, and examined under a compound microscope. The right ovary was also removed from every animal and prepared for microscopic study.

[1] Aided by a research grant from the University of California. The writer wishes to express his appreciation to Dr. B. M. Allen for criticisms and suggestions and to Mr. Allen Lein for valuable assistance.

RESULTS. The removal of the thyroid gland 1½ hours to 12 days after mating had no appreciable effect on the length of the period of gestation in the rabbit. Since in both control and experimental animals the duration of pregnancy varied from 29 to 33 days, the slight difference between the average length of the gestation period for the controls (30.7 days) and the thyroidectomized (31.1 days) rabbits was not significant. Moreover, no histological differences were observed in the ovaries from both groups, large and small follicles being noted in all.

All experimental animals were shown to be hypothyroid as is evidenced by their decreased rate of oxygen consumption, which varied from 14 to 38 per cent (average, 29 per cent) below the average rate of oxygen consumption for the 15 control animals. No correlation existed between the rate of oxygen consumption and the length of the gestation period.

Examination of the entire thyroid region of all operated animals revealed that 8 animals possessed thyroid remnants. These exhibited an oxygen consumption rate varying from 14 to 26 per cent below that of the controls.

It was observed that 7 of the 52 experimental animals did not pass through a normal delivery. Two were found dead 33 days after mating and one, 31 days post coitum. These contained from 5 to 9 dead fetuses in utero. The embryos appeared normal in size and development. The remaining four rabbits delivered within the normal limits of gestation but after parturition exhibited a paralysis of the hind legs. Two of these paralyzed animals died within three days; the other two recovered after approximately 10 days.

DISCUSSION. It is apparent that in the rabbit, thyroid ablation does not interfere with the length of the period of gestation, nor does it alter the histology of the ovary. Moreover, 14 of the thyroidectomized animals were mated again after their first delivery and passed through a second successful pregnancy in spite of the absence of a thyroid. These findings are not in accord with those of Ukita (1920) who reported an increase in the length of the gestation period of over 100 per cent in rabbits after the removal of the thyroid.

Seven thyroidectomized rabbits refused to mate after delivery even though attempts to mate them were made three times weekly for 6 weeks. A laparotomy was done on these animals to eliminate the possibility of pseudopregnancy. In one, the uterine horns were small and pale but in the other six animals the horns were slightly hyperemic. There were large clear follicles near the surface of the ovaries and no corpora lutea or corpora hemorrhagica were observed. The incisions were closed and after one week had been allowed for recovery, the rabbits were injected intravenously with 1 cc. of a sheep pituitary extract prepared after the method of Van Dyke and Wallen-Lawrence. Twenty-four hours later a second laparotomy was done. Ovulation had occurred in all animals,

indicating that thyroidectomy did not influence the potential ability of the ovaries to react normally to the stimulus of an anterior pituitary substance.

The relatively high percentage of rabbits that either died at the time of delivery or exhibited paralysis of the hind limbs immediately after delivery suggests that the procedures employed interfered in some way with the normal processes of parturition. It is probable that the animals affected may have suffered a diminution in the normal supply of parathyroid hormone since no attempt was made to preserve the glands.

SUMMARY

1. Thyroid removal from 1½ hours to 12 days after mating does not alter the length of the period of gestation in the rabbit nor does it alter the histology of the ovaries.

2. There is no correlation between the rate of oxygen consumption and the length of the period of pregnancy.

3. Fourteen thyroidectomized rabbits were mated again after their first delivery and successfully terminated a second pregnancy within the limits of a normal gestation period.

4. It is suggested that the parathyroid hormone may be important in the normal processes of parturition.

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RELATION OF THE VARIOUS GROUPS OF THE ADRENALIN MOLECULE TO ITS INTESTINE INHIBITING FUNCTION IN UNANESTHETIZED DOGS[1]

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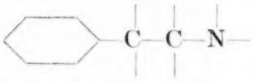
The wall of the intestine is composed in part of smooth muscle which is inhibited by adrenalin, "intestinal sympathin," and its adrenergic nerve supply (1, 2, 3). Additional facts are necessary before the exact nature of the chemical mediator and the sympathins produced at inhibitory adrenergic endings can be determined. In this study equivalent intestine-inhibiting injection rates have been determined in unmedicated dogs for six compounds which differ from adrenalin by lacking one or two of the groups that distinguish adrenalin from the fundamental sympathomimetic nucleus designated by Barger and Dale (4)



A comparison of the results obtained with the results of quantitative studies of the same compounds as stimulators of the nictitating membrane (5, 6) serves to accentuate differences in the reception of the compounds by the two types of smooth muscle.

METHODS. Records of the motility of jejunal loops made into Thiry or Thiry-Vella fistulae were taken by the balloon-mercury-manometer method. Six dogs were used. Three of these each had one innervated and one denervated Thiry fistula. Three dogs had innervated Thiry-Vella fistulae, one of which was subsequently denervated. Methods of preparing and denervating fistulae have been previously described (1).

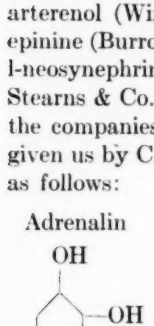
Intravenous injections at various constant rates were made by means of an electric motor geared to a screw which moved the plunger of a syringe at the desired rate. The rate of injection could be increased by any increment from 1 to 4 cc. per minute. For each compound a dilution was found which was near the threshold for producing a short period of inhibition of the intestine when injected at a rate of 1 cc. per minute. Injections were maintained for a period of from one to eight minutes. Acidified stock solutions of the compounds were kept in the refrigerator.

[1] Aided in part by a grant from the John and Mary R. Markle Foundation.

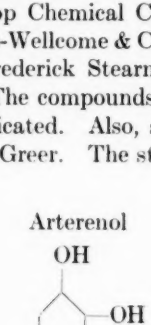
Solutions for injection were prepared by introducing the necessary amount of the stock solutions into 100 cc. volumetric flasks and filling to the mark with physiological saline buffered at a pH of 4.9 to 5. No solutions were used which showed discoloration. No loss of potency was found in the stock solutions which remained colorless. Injection of the diluent alone had no effect on intestinal motility.

The compounds studied were l-adrenalin (Parke Davis & Co.), dl-arterenol (Winthrop Chemical Co.), cobefrin (Winthrop Chemical Co.), epinine (Burroughs-Wellcome & Co.), kephrine (Alba Pharmaceutical Co.), l-neosynephrin (Frederick Stearns & Co.), and dl-synephrin (Frederick Stearns & Co.). The compounds were furnished through the courtesy of the companies indicated. Also, some di-nor-epinephrine (arterenol) was given us by C. M. Greer. The structural formulae of the compounds are as follows:

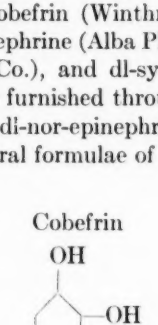
Adrenalin



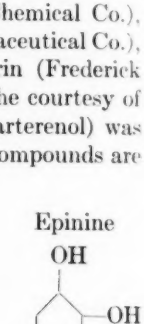
Arterenol



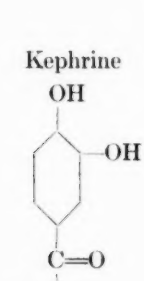
Cobefrin



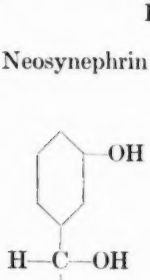
Epinine



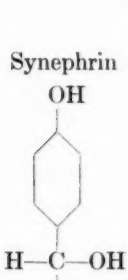
Kephrine



Neosynephrin



Synephrin



Some interpretations of the data obtained are based on the view that the compounds inhibit by direct action on the smooth muscle of the intestinal wall and not indirectly by ischemia resulting from vasoconstriction. The possibility of reduced blood supply being a factor requires consideration, since severe ischemia of the intestine inhibits its motility (7). Several facts indicate that reduced blood supply is not the factor responsible for inhibition of the intestine in these experiments. 1. The most marked ischemia possible, such as may be obtained by obstructing the aorta, requires at least 8 seconds before producing inhibitory effects (7). Ischemia following intravenous injection could not begin to develop before one circulation time (at least 12 sec.). Therefore, even if maximal, ischemia could not be a factor until at least 20 seconds after the beginning of the injection. However, injections of the amines typically produce maximal inhibitory effects within 12 to 15 seconds. 2. The inhibition of the denervated intestine is produced by some of the compounds when the injection rate is below threshold for blood pressure effects. 3. Plethysmograph records show that the intestinal motility will remain inhibited following an adrenalin injection even after such records indicate that the intestine has become hyperemic. 4. One of the best vasoconstrictors of the group is one of the poorest inhibitors of the intestine. 5. The effects of the compounds are qualitatively the same as the effects of sympathetic nerves to the intestine, an effect shown by Bayliss and Starling (7) to be independent of vasoconstrictor action. 6. The effects of the compounds on the intact intestine are qualitatively identical to the effects of adrenalin on the isolated intestine. It is considered, therefore, that the technique employed permits the study of the response to sympathomimetic compounds of a single effector, namely, smooth muscle which is inhibited by its adrenergic nerve supply. The advantages of the technique, when both innervated and denervated segments of intestine are used, are similar to those listed by Bacq (5) for the nictitating membrane. Among these advantages are ease of recording, relative ease of denervation, great sensitivity, and regularity of response to equivalent dosage. In addition, anesthesia is not necessary, and the same indicator may be used for the entire series of compounds.

The compounds, with two exceptions, characteristically produce effects on intestinal motility within a period of less than two circulation times. This fact indicates that time for building up a concentration in the blood was not required. All of the compounds, except cobefrin, are of the general formula which has been shown to be susceptible to rapid enzymatic destruction in tissues (8). Moreover, Rogoff (9) has found that continuous intravenous injections of adrenalin at rates several times as fast as any used in these experiments is not accompanied by an increased adrenalin content of the blood recovered from the adrenal vein. There is nothing to indicate that the differences in injection rates are not an index

to the differences in the concentrations reaching the intestine except in the case of cobefrin and the two least potent compounds. The latter apparently must be injected rapidly enough to exceed the rate of enzymatic destruction.

RESULTS. *Qualitative effects of the compounds.* All of the compounds were found to inhibit the rhythmic contractions and decrease the tonus of either the innervated or the denervated intestine when given in a concentration sufficient to produce any effects on the intestine (figs. 1–7). No injection rates were found which caused an increase in the motility of the intestine *during* the injection. Hyper-motility commonly occurred during the recovery phase following the end of the injection. This recovery phase may be the most conspicuous feature of the record when the injection is very short.

The characteristic effects on intestinal motility are as follows. 1. After a short latent period equivalent to one or more circulation times there appears a reduction in the amplitude of rhythmic contractions and a decrease in tonus. The rapidity of onset of the inhibition produced by a given compound is dependent on the injection rate. Of a total of 346 injections 83 were below the threshold for producing effects on intestinal motility. Of the remaining 263 injections at various rates ranging from barely threshold to five or six times the threshold rate the period from the beginning of the injection to the beginning of inhibition had a duration of 12 to 20 seconds in 47 per cent of the injections, 21 to 30 seconds in 18 per cent of the injections, and longer than 40 seconds in 15 per cent of the injections. One-half of the latter occurred with injections of synephrin or cobefrin. A building up of the concentration of these two compounds in the blood during continuous injections is to be expected,—synephrin because of the high concentration which must be injected, and cobefrin because it is not susceptible to enzymatic destruction (8). It is very likely that these compounds, and possibly neosynephrin, are less potent than the figures obtained by constant injection methods would indicate. 2. If the injection of any one of the compounds, other than synephrin, cobefrin, and neosynephrin, be continued at a rate two or three times that required to produce initial complete inhibition, a "breaking through" the inhibition occurs after a few minutes. The intestine tends to recover normal motility during the continuation of the injection. The breaking through appears later with the higher injection rates. Therefore, the duration as well as the degree of inhibition varies with the rate of injection used. Breaking through does not appear as readily with some of the compounds as it does with adrenalin. 3. When the injection is stopped, the intestine begins to recover from the inhibitory effects of the compound within a circulation time or two. The amplitude of rhythmic contractions shows a gradual increase to "normal" or greater. A tonus wave commonly accompanies

the hyper-motility. In general, the hyper-motile recovery phase is more likely to occur in the denervated than in the innervated intestinal segments.

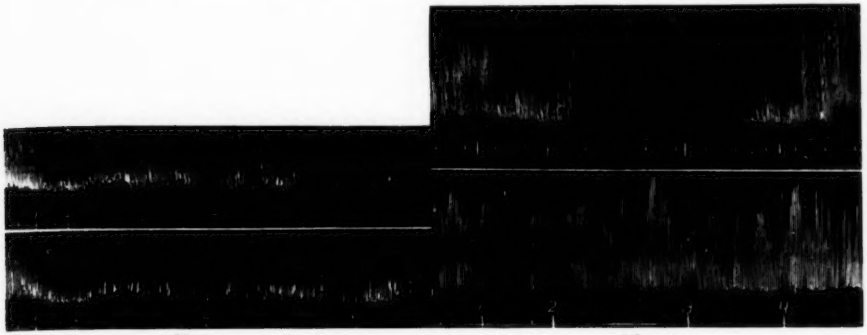


Fig. 1 Fig. 2

Fig. 1. Effect of adrenalin and arterenol on the motility of the innervated Thiry-Vella fistula of dog 4a. Upper record shows effect of adrenalin 1:250,000 injected at a rate of 1 cc. per minute between 1 and 2, 2 cc. per minute between 3 and 4, and 4 cc. per minute between 5 and 6. Lower record shows effect of arterenol 1:100,000 injected at a rate of 2 cc. per minute between 1 and 2, 1¼ cc. per minute between 3 and 4, and 4 cc. per minute between 5 and 6. Dog weighed 9 kilo. Time in minutes.

Fig. 2. Effect of arterenol 1:500,000 on the denervated (upper record) and the innervated (lower record) intestinal segments of dog 1. Injection rate 2 cc. per minute between 1 and 2, 4 cc. per minute between 3 and 4. Dog weight 19 kilo. Time in minutes.

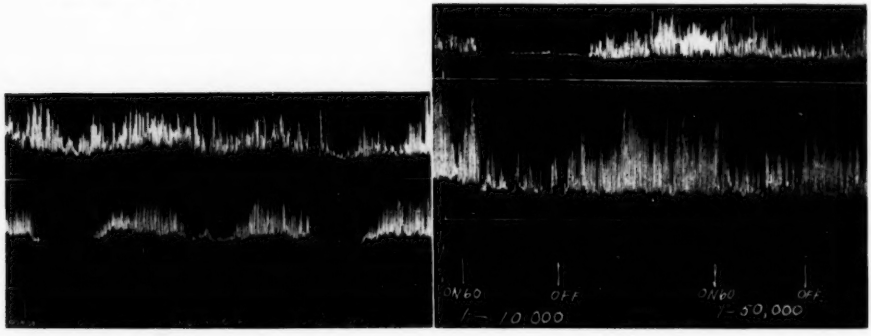


Fig. 3 Fig. 4

Fig. 3. Effect of cobefrin 1:100,000 on the denervated (lower record) and the innervated (upper record) intestinal segments of dog 1. Injection rate 2 cc. per minute at ON 30, 1 cc. per minute at ON 60, and 4 cc. per minute at ON 15. Dog weight 19 kilo. Time in 10 second and 1 minute intervals.

Fig. 4. Effect of epinine on the motility of the denervated (upper record) and the innervated (lower record) intestinal segments of dog 1. Injection rates were 1 cc. per minute of 1:10,000 followed by 1 cc. per minute of 1:50,000. Dog weight 19 kilo. Time in 10 second and 1 minute intervals.

If a given segment shows a hyper-motile recovery phase following adrenalin injection, it ordinarily shows it also when recovering from the effects of the other compounds. When a second injection, identical in rate to the first,

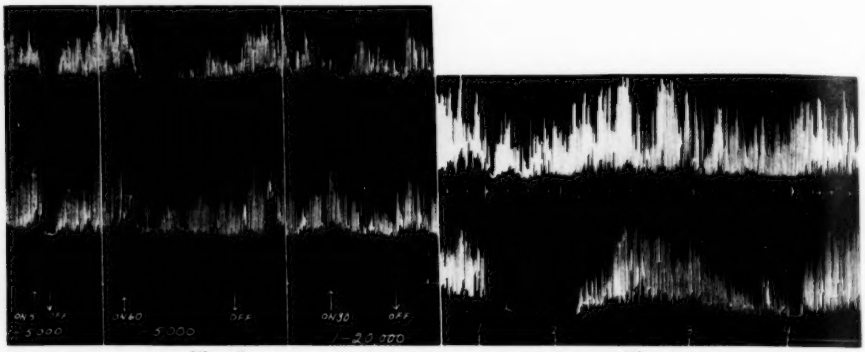


Fig. 5 Fig. 6

Fig. 5. Effect of kephrine on the denervated (upper record) and innervated (lower record) intestinal segments of dog 1. Injection rates were 4 cc. per minute of 1:5000 (left), 1 cc. per minute of 1:5000 (middle), and 2 cc. per minute of 1:20,000 (right). Dog weight 19 kilo. Time in 10 second and 1 minute intervals.

Fig. 6. Effect of neosynephrin 1:10,000 on the innervated (upper record) and the denervated (lower record) intestinal segments of dog 3. Injection rates were 4 cc. per minute between 1 and 2, 2 cc. per minute between 3 and 4. Dog weight 14.5 kilo. Time in minutes.

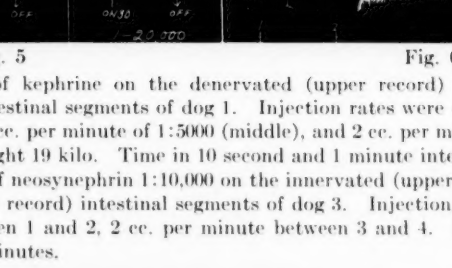


Fig. 7. Effect of synephrin 1:250 on the innervated (upper record) and the denervated (lower record) intestinal segments of dog 3. Injection rates were 2 cc. per minute at ON 30 and 1 cc. per minute at ON 60. Break in record represents a 14 minute interval. Dog weight 14.5 kilo. Time in 10 second and 1 minute intervals.

is begun during the recovery phase, the inhibitory effects resulting from the second injection are less than those resulting from the first.

It has already been shown that the effects on the intestine of reflexly

activated adrenergic nerves, or of the sympathin produced by them, are indistinguishable from the effects of adrenalin (2). However, the intestinal effects of adrenalin are not characteristic of adrenalin alone. Since the mediator of impulses at adrenergic intestinal nerve endings is considered to be adrenalin or a similar compound, it appears unlikely that these nerves could ever perform anything but an inhibitory function.

Sensitization by denervation. Hypersensitivity to sympathomimetic amines has been amply demonstrated for smooth muscle of the type that contracts in response to adrenalin (10). Hypersensitivity to adrenalin has been demonstrated in the intact dog for smooth muscle of the type that is relaxed by adrenalin (2). The denervation of intestinal smooth muscle sensitizes it also to the inhibitory effects of each of the compounds studied. Sensitization is illustrated in figures 2 to 7. These records were taken simultaneously from innervated and denervated fistulae in the same animal. For each of the three animals having both innervated and denervated fistulae an injection rate for any one of the compounds could be determined which would produce complete inhibition of the denervated segment without significantly affecting the innervated one. The sensitivity of the intestine is increased by the postganglionic denervation so that it is 2 to 8 times more sensitive than normal. In one animal the sensitivity of a short Thiry-Vella fistula was studied. The loop was then denervated by doing a laparotomy between the two ends, lifting the mesenteric pedicle on a rod, and cutting everything in the pedicle except an artery and two veins. The vessels were then cleaned of nerve fibers and painted with concentrated phenol followed by alcohol and saline. Records taken between 7 and 14 days after this operation showed that complete sensitization with regard to all of the compounds had been attained, the segment being 4 to 8 times as sensitive as before the denervation. The sensitivity to adrenalin was recorded during the next few weeks, and no further increase in sensitivity occurred. Sensitization of the intestine develops fully as rapidly as the sensitization of the nictitating membrane (10).

Absolute and relative intestine-inhibiting potency. The lowest adrenalin injection rate sufficient to produce a few seconds of complete inhibition in the four denervated segments studied was between 0.0001 and 0.0002 mgm. per kilo per minute. This is about one-half the minimal pressor dose reported by Dragstedt et al. (11) for dogs under similar conditions. The threshold inhibitory dose for the other compounds can be calculated by multiplying the figures in table 1 by these values.

In table 1 the extremes are listed for the increase in injection rates that were necessary for each of the compounds in order to produce a degree of intestinal inhibition comparable to that produced by test doses of adrenalin. Results are tabulated for seven indicators. The reciprocal of the number

indicates the potency of the corresponding compound as compared to that of adrenalin. As previously stated under methods, cobefrin, synephrin, and neosynephrin may be less potent than indicated.

Various authors (6, 12) have emphasized the importance of the meta —OH group of the adrenalin molecule (lacking in synephrin) to certain sympathomimetic functions of adrenalin. Of the groups of the adrenalin molecule that distinguish it from the fundamental nucleus the meta —OH group is the most important for the intestinal smooth-muscle-inhibiting function of adrenalin. Next in order are the para —OH, the secondary alcohol —OH, and least important, the $—CH_3$ on the nitrogen atom. When the order of potency of these compounds as intestinal inhibitors is compared with the order for promoting contraction of the nictitating

TABLE 1

Number of times the injection rates for each of six compounds must be increased beyond that of a test dose of adrenalin in order to duplicate the degree of inhibition of intestinal motility produced by the adrenalin

Figures for relative potency of the compounds as contractors of the nictitating membrane* are listed in the last column

	DOG 1, DEN.	DOG 2, DEN.	DOG 3, DEN.	DOG 4b, DEN.	DOG 4a, INN.	DOG 5, INN.	DOG 6, INN.	EXTREMES	RATIO FOR n.m.
Adrenalin (l)	1	1	1	1	1	1	1	1	1
Arterenol (dl)	1½–4	1½–3	2	2½–3	2		2–2½	1½–4	10–30
Cobefrin	2½–5	5–10	3½	5–7	5	10	3–5	2½–10	
Epinine	10	10–20	10	10	10		15–25	10–25	20
Kephrine	50–100	50–100	33	25	40		50	25–100	75
Neosynephrin (l)	100	100	50	25	40	25	25–50	25–100	3½–4
Synephrin (dl)	1,000	1,700	660	2,500	1,500	2,500	1,000	660–2,500	30–120

* Data from Bacq (5, 6).

membrane (5, 6), neosynephrin is placed fourth or fifth in the former case and second in the latter. Cobefrin is not included in the comparison. Arterenol and epinine, markedly more potent than neosynephrin as intestinal inhibitors, are considerably less effective than neosynephrin as contractors of the nictitating membrane. It is clear that no consistent relationship exists between the smooth-muscle-relaxing (intestine) and the smooth-muscle-contracting (nictitating membrane) properties of these amines. Such a result is in accord with the postulation of at least two types of smooth muscle with regard to "receptive mechanisms." A given group of the adrenalin molecule, as the para —OH, bears a more important relationship to one type of smooth muscle than to the other type, while another group, as the meta —OH, is necessary for a high degree of potency in both types. It cannot be expected, therefore, that the order of potency for a mixed response, such as blood pressure changes, will be consistent

with either type of single-effector response. On the other hand, if there are only two physiological types of smooth muscle, it seems that the agents should show the same order of potency for one of these types regardless of the organ in which it is located. The importance of the catechol nucleus for a high degree of bronchodilator efficiency in dogs has been emphasized (13), neosynephrin being classified as a poor agent for this purpose. The catechol nucleus is also necessary for a high intestine-inhibiting potency, since the two least effective compounds of the series are those which lack one or the other of the phenolic —OH groups.

It would be of value from the standpoint of therapy to establish the physiological classification of smooth muscle and the potency of the various amines with regard to the physiological types of this muscle. Then a compound having the desired property highly developed, but lacking the other property, could be used instead of adrenalin which is highly effective on both types.

In connection with arterenol some facts may have a bearing on the suggestion of Greer et al. (14) that it be assumed as a working hypothesis that l-arterenol (l-nor-epinephrine) is the sympathin that comes into circulation from effectors that are stimulated by their adrenergic nerves. Greer describes this sympathin (S_c) as an agent that may cause relaxation or contraction, depending on the effector cell concerned, but having a greater intrinsic power of producing contraction and less intrinsic power of producing relaxation than the other sympathin (S_r) which is, according to the theory, l-adrenalin. In experiments in which the response may be considered to be that of a single type of smooth muscle, arterenol is less potent than adrenalin whether the effector shows a motor response, such as the retractor penis (4) and nictitating membrane (5), or an inhibitory response, such as the non-pregnant uterus (4), the intestine, and the bronchioles (12). The difference in potency of adrenalin and arterenol is less with regard to the intestine-inhibiting function in the dog (see fig. 1) than it is for any of the other single-effector responses. This is especially true if it may be assumed that the laevo form of arterenol would show greater intestine-inhibiting potency than the racemic form which was used. If it should be proved that a sympathin is a compound of less potency than adrenalin, it seems more logical to assume in the light of present facts that the mediator is adrenalin, and the *less potent* sympathin is a stage in the destruction of the mediator.

SUMMARY

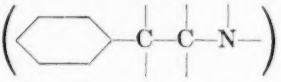
Equivalent intestine-inhibiting injection rates have been determined in unmedicated dogs for arterenol, cobefrin, epinine, kephrine, neosynephrin, and synephrin. The use of these compounds has permitted evaluation of the relative importance of the various groups of the adrenalin molecule

to its intestine-inhibiting function and, perhaps, to its smooth muscle inhibiting function in general.

The effects of the compounds on intestinal motility are qualitatively similar. These consist of decreased tonus and inhibition of rhythmic contractions.

Within one to two weeks after postganglionic denervation the intestine becomes two to eight times more sensitive to each of the compounds than it was before denervation.

Removal of any one of the groups of the adrenalin molecule that distinguish it from the fundamental sympathomimetic nucleus



results in a compound of less intestine-inhibiting potency. The most important single group is the meta —OH. Next in order are the para —OH, the secondary alcohol —OH, and least important, the $-CH_3$ on the nitrogen atom.

All of the compounds used both inhibit the intestine and stimulate the nictitating membrane (5, 6). A comparison of the importance of a given group of the adrenalin molecule to its intestine-inhibiting function with the importance of the same group to its nictitating membrane-stimulating function indicates that no consistent relationship exists between the smooth muscle-relaxing and smooth muscle-contracting potency of these amines.

The relation of the above facts to sympathin theories and the physiological classification of smooth muscle is discussed.

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VARIATIONS IN INTRAMUSCULAR PRESSURE DURING POSTURAL AND PHASIC CONTRACTION OF HUMAN MUSCLE[1]

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In 1936 Henderson *et al.* described a method for determining the tonus of human skeletal muscle by measuring intramuscular pressure. The procedure was subsequently modified in detail and successfully applied by Kerr and Scott (1936) and Beiglbock and Junk (1937). Youmans and his associates had developed independently a method only slightly different in principle. Their first report (Wells, Youmans and Miller, 1938), based upon a six year experience in its use, led them to conclude that intramuscular pressure serves as an unreliable criterion of tonus. In the meantime we had repeated Henderson's observation on the postural and voluntary contraction of the human gastrocnemius in various stances. It was our object to determine whether intramuscular pressure is a practical method for the quantitative study of the distribution of tonus between various antigravity muscle groups during normal standing.

METHODS. Intramuscular pressure was determined by Henderson's technique. Precision was added by the use of a mercury reservoir supported from a rack and pinion for the production of smooth and rapid changes in pressure in the saline containing glass capillary to which the needle is attached (Beiglbock and Junk, 1937). Deformation of the meniscus and minute movements of the saline column were observed through a magnifying glass against a suitable ground. Pressure changes were read from a bromoform manometer.

The method was first validated by determining the intramuscular pressure of artificially stimulated frog and human muscle. The frog was pithed; leaving the muscular attachments intact, nerves to the quadriceps, gastrocnemius and hamstrings were dissected free and stimulated with tetanic currents of graded strength. In man only the biceps was stimulated, using the unipolar method. Figures 1a and 1b illustrate the character of the results obtained from 23 experiments on the frog and 6 on 2 human subjects.

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The term "quiet standing" is a misnomer implying constancy in the status of the supporting muscles. We have shown that the center of gravity of the body as a whole shifts incessantly during natural, comfortable standing (Hellebrandt and Braun, 1939) and that the amount of movement in a relaxed subject may be extreme (Hellebrandt, 1938). The pattern of the proprioceptive bombardment of motor neurons must be a replica of the oscillogram of the shifting center of weight, fresh stretch afferents being stimulated with each new position of the center of gravity projection within the confines of the base. Constancy of intramuscular pressure in

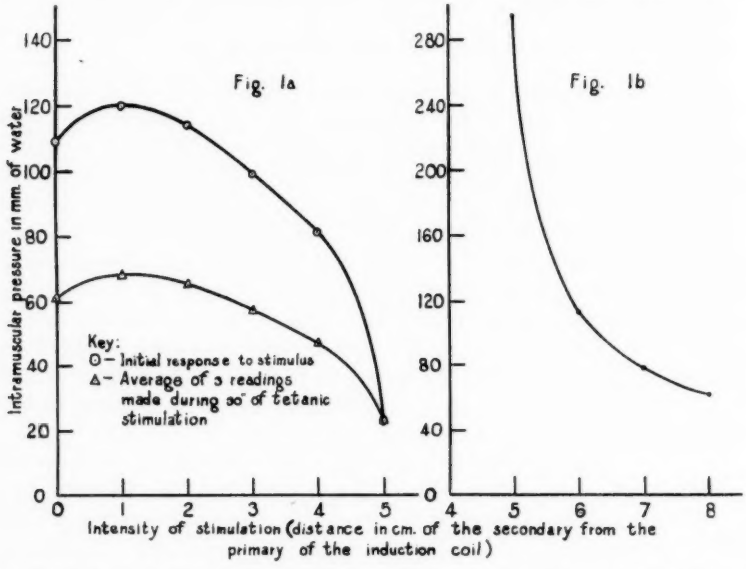

Fig. 1a. The relation of intramuscular pressure to intensity of stimulation. (Frog gastrocnemius.)

Fig. 1b. The relation of intramuscular pressure to intensity of stimulation. (Human biceps.)

the anti-gravity muscles during standing can be expected theoretically only if the center of gravity of the body as a whole remains immobile, a condition never attained even by vigorous young adults. Wells, Youmans and Miller (1938) have observed that there is even considerable variability in the intramuscular pressure of the relaxed recumbent subject. It seemed to us, therefore, that the tonus of a given muscle, particularly during standing, could be judged only from the *general level* of repeated observations made at short intervals of time. Youmans *et al.* (1938) had shown that several cubic millimeters of fluid may be injected into muscle during the

course of an experiment without any rise in local pressure provided the fluid continues to flow freely both out of and back into the capillary tube system. Since the equilibrium point may be determined by minute movements of the column in the capillary tube following visible deflection of the meniscus, there appears to be no contraindication to this modification of Henderson's procedure.

The reliability of the technique was checked by repeated determinations on the relaxed and voluntarily contracted biceps of 2 subjects. Figure 2 shows the variability of successive observations in relation to the mean intramuscular pressure of the relaxed biceps. The range during 15 minutes did not exceed 0.2 cm. of water, a degree of variation which cannot be considered excessive in the light of the limitations of the method and difficulties of maintaining complete relaxation by voluntary effort. Maximal contraction elevated the intramuscular pressure from a mean value of 110 mm. of water to 150 in one subject and from 90 to 198 in the other.

In our hands the procedure was not easily applied to the standing subject. The peculiarly sickening sensation produced by the contraction of the gastrocnemius on the needle to which Youmans *et al.* refer was poorly tolerated, necessitating the termination of a number of experiments. There were several cases of syncope. All standing observations were limited to 2 trained subjects. Using sterile precautions, a no. 22 hypodermic needle was thrust to its full length into the belly of the muscle after novocaine anesthesia of the skin. Where there is considerable movement we found this technique more satisfactory than the use of a large, long needle inserted to only a portion of its full length. Except in one experiment, observations were limited to the gastrocnemius. The subject stood unsupported on the platform of the center of gravity apparatus previously described (Kelso and Hellebrandt, 1937; Hellebrandt and Kelso, 1938). Oscillograms were made of the shifts in the center of weight in the two vertical orientation planes during successive 60 second periods of various stances. In the mid-interval of each, intramuscular pressure was read.

RESULTS AND THEIR DISCUSSION. Evidence for the validity and reliability of the procedure has already been presented in figures 1 and 2. Comparable results on the relaxed biceps are given by Henderson *et al.* (1936), Kerr and Scott (1936) and Beiglbock and Junk (1937). Ours tend to be somewhat higher. Wells, Youmans and Miller (1938) observed widely varying maximal values of intramuscular pressure during the voluntary contraction of different muscles in man. For the same stimulus the hamstrings of the frog developed less pressure than the antigravity extensors upon which hopping depends.

1. Variations in intramuscular pressure in the gastrocnemius during relaxed standing. These are shown in figure 3. The range is many times

that observed in successive minutes on the relaxed biceps. It averaged 13.4 cm. of water in the four standing experiments presented. In the one showing the least variability (expt. 3) the difference between the maximum and minimum pressures recorded is more than 30 times that observed in an equal interval of time (15 min.) on the resting biceps.

The mean level of intramuscular pressure also shows considerable variation on the four different days, confirming the findings of Wells, Youmans and Miller (1938). They observed pressures below 50 mm. of water in the gastrocnemius of the normal, relaxed subject in the recumbent posture. The average of the 40 determinations of standing intramuscular pressure

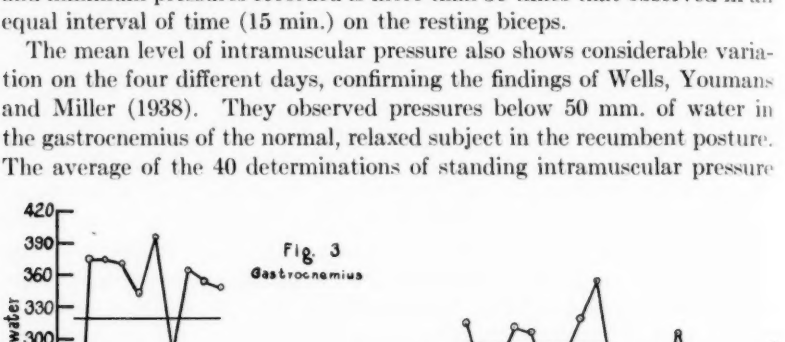

Fig. 2. Variation in intramuscular pressure in the relaxed biceps. (L. E. A. K.)
Fig. 3. Variation in intramuscular pressure in the gastrocnemius during relaxed standing. (F. A. H.)

recorded in figure 3 is 264 mm. of water, or 5 times the resting value. The observations on the second subject were similar, the mean level of standing pressure being 244 mm. of water, ranging between 135 and 355, a variability which exceeds that of the data presented in the illustration.

2. Variations in intramuscular pressure in the gastrocnemius in different stances. The subject assumed a relaxed, normal comfortable stance on the center of gravity apparatus. The constancy of the posture was judged by similarity in the minute to minute center of gravity oscillograms. The eyes were then closed. This increases postural sway through the loss of impulses over the visual route which normally augment tonus. The oscillograms increased, spreading in both vertical orientation planes. The

weight was then shifted to the leg without the needle. The center of gravity projection moved from its normal location in the center of the base to an eccentric position approaching the lateral margin of static security on the side of the weight bearing leg. Attempts to hold the full weight on the needle bearing extremity were resisted because of pain but the weight could be held on the toes with knees flexed and then fully extended. The latter postures were each maintained for only 2 or 3 minutes. The mean level of intramuscular pressure in the right gastrocnemius during this series of stances is presented in figure 4a. Increase in postural sway when the eyes are closed, theoretically produced by a loss of tonus, is associated with a fall in intramuscular pressure which sinks to a still lower level when the weight is shifted away from the needle bearing leg. Extension at the ankle joint is associated with a significant rise in intramuscular pressure. When full extension of the knees is added, the intramuscular pressure is further augmented. In Henderson's experiment the intramuscular pressure in the gastrocnemius rose from 143 to 312 mm. of water when the weight was held on the toes. We observed it to rise from 285 to 338 and from 230 to 343 in two similar experiments, increasing to 401 and 407 respectively when the knees were fully extended.

3. Shifts in tonus between the gastrocnemius and quadriceps during steady and relaxed standing. An introspective subject is conscious of a shifting of tension between ankle, knee and hip joint extensors during prolonged comfortable standing, one group or another apparently bearing the brunt of the stabilizing work at a given time. In the light of this subjective observation, simultaneous determinations of intramuscular pressure were made on the quadriceps and gastrocnemius during 2 diametrically opposed types of standing, a relaxed stance with the eyes closed and during the steadiest posture attainable by strong voluntary effort. The mean intramuscular pressures during 5 minutes of fixed standing followed by 10 minutes in a relaxed posture and a return to a rigid stance for another 5 minutes are presented in the column diagram, figure 4b. The multijointed weight bearing limbs are supported by stabilizing bands placed on alternate sides of the extremity. The data indicate that the more centrally placed guy rope is taut when the limb as a whole is held rigid in voluntary, steady standing. Intramuscular pressure is higher in the quadriceps than in the gastrocnemius. When the stance is relaxed, the peripheral guy rope catches the swaying limb and intramuscular pressure is higher in the gastrocnemius than in the quadriceps. A return to a rigid stance reverses the distribution of tension. These observations suggest that the rhythmic variability in intramuscular pressure in one antigravity muscle may be due in part at least to a reciprocal sharing of the work of standing by the different antigravity groups.

If tonus is due to an asynchronous rotation of contraction between motor units in response to an incessantly shifting stretch stimulus, it remains to

be determined whether the resultant, short lasting changes in intramuscular pressure are faithfully portrayed by a recording system variously remote from the anatomical parts concerned and separated from them by barriers offering differing degrees of resistance, some of them in a constant state of flux. Wells, Youmans and Miller (1938) present arguments against the validity of this concept. However, there is little doubt that as the contraction of motor units become more synchronous the pressures accrue sufficiently to reach values much in excess of the normal turgor present in the tissues. It is probable that in short time experiments concerned with relaxed and comfortable standing, the number of motor units contracting

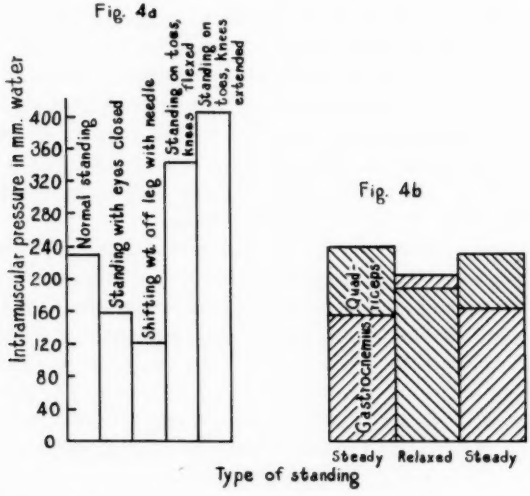

Fig. 4a. Variation in intramuscular pressure in the gastrocnemius in different stances.

Fig. 4b. Shifts in tonus between the gastrocnemius and quadriceps during steady and relaxed standing.

and the synchronicity of their response are factors of greater importance in the production of intramuscular pressure changes than stasis or edema.

These and our previous experiments on standing suggest that the differentiation of postural from phasic contraction of the anti-gravity muscles on the basis of the presence or absence of movement of the levers to which they are attached and the duration of response is hardly justifiable. Postural contraction appears to be composed of numerous quick movements associated with sharp changes in intramuscular pressure too small to be detectable by ordinary means and hence giving the appearance of smooth and steady holding of various postures. Standing is indeed movement upon a stationary base and postural contraction the resultant of a complex

pattern of brief, quick, maximal contractions of small numbers of motor units probably in response to constantly changing stretch stimuli of variable intensity. Our methods of converting shifts in the center of gravity of the body as a whole into gravitational rotatory stresses effective at specific joints are not yet sufficiently precise to permit a correlation between intramuscular pressures and the stresses being equilibrated at the instant of their determination.

SUMMARY AND CONCLUSIONS

The validity of the intramuscular pressure method of measuring tonus was determined on frog and human muscle stimulated by tetanic currents of various strengths. The reliability of the technique was tested on the relaxed biceps. Intramuscular pressure readings were made on the human gastrocnemius stimulated by the gravitational rotatory stresses of postural sway and during voluntary weight-bearing contraction. In one experiment synchronous observations were made of intramuscular pressure in 2 anti-gravity muscles, the gastrocnemius and quadriceps. The data presented lead us to conclude that:

1. Gross changes in tonus are reflected by significant variations in intramuscular pressure.

2. Intramuscular pressure is lowest in the relaxed muscle, higher during postural contraction and maximal during voluntary effort.

3. Intramuscular pressure in the relaxed muscle is relatively constant in contrast with the marked, rhythmic variability which occurs during postural contraction.

4. The rhythmic variation of intramuscular pressure in the gastrocnemius during comfortable relaxed standing is probably due to a shift in the incidence of tension between different antigravity groups.

5. The method is not easily applied to stances associated with excessive postural instability.

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EFFECT OF AVITAMINOSIS A ON THE HUMAN BLOOD PICTURE

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During the past few years there has been a shift in the center of interest from the fully developed vitamin A deficiency disease to the mild or latent forms. The former is uncommon, easily recognized, and usually responds promptly to vitamin therapy. On the other hand, there is evidence that mild or minimal vitamin deficiency is more frequent, and therefore constitutes a more serious problem than the rare, advanced cases. In the past, the recognition of mild cases has been difficult and uncertain; and especially was this true when this deficiency appeared as a complication of other diseases. The most readily appreciated clinical symptoms of vitamin A deficiency have to do with changes occurring in the eye. Of the several recognizable symptoms of eye changes, it now appears that night blindness, or the inability to see well in dim light, is the earliest, while xerophthalmia, xerosis or keratomalacia, all of which involve various degrees of keratinization of the epithelium of the eyeball, lids, and the paraocular glands, represent the more advanced stages. Fortunately, however, there are available today several methods which, used either singly or together, may prove of value in the diagnosis and study of the mild forms of vitamin A deficiency. The visual photometer or "adaptometer" has been found to be reliable and accurate in measuring small differences in sensitivity to dim light, while light scrapings of the bulbar conjunctiva show the presence or absence of cornified or keratinized epithelia.

In 1937 Abbott and Ahmann found that rats, fed a vitamin A deficient diet until gross symptoms of avitaminosis appeared, had a differential leucocyte count which varied from the normal hematologic standard in several respects. The blood picture of these rats showed a decrease in polymorphonuclears, an increase both in large lymphocytes and in young cells and a general degeneration, especially of the granulocytes. When large amounts of vitamin A were administered daily for several days there was a gradual improvement in the general condition, and in a few weeks the differential count was considered normal. From this work it was concluded that the differential leucocyte count offered a convenient and accurate method of diagnosing avitaminosis A in rats.

The plan of this study was to make differential white cell counts on the blood of individuals whose diets and symptoms indicated a vitamin A deficiency. If the blood picture of these subjects was similar to that of rats fed a vitamin A deficient diet, and if, upon the administration of vitamin A, the blood picture became normal, the differential count would then become of diagnostic value in detecting varying degrees of vitamin A deficiency in man.

METHODS. *Selection of subjects.* The subjects making up the experimental groups consisted of college students, women, and rural school children. For various reasons these individuals had been living on restricted diets for periods which varied from a few months to many years.

Laboratory tests. Each subject was given a physical examination, and blood was taken for total red and white cell counts, hemoglobin estimations, and differential counts. Venous blood was used in all tests. The total counts were made with a Levy-Hausser counting chamber with Neubauer ruling and the percentage of hemoglobin determined with a Dare hemoglobinometer. The differential white blood cell counts were based on 300 cells on fixed smears stained with Wright's stain. In making these counts, 100 cells on each of three cover glasses were counted. The Washburn method for the peroxidase reaction was used to distinguish young lymphocytes from young granulocytes.

If the blood picture indicated a vitamin A deficiency, large doses of vitamin A were administered daily and the effect on the blood picture noted at ten-day intervals.

Dietary histories. Individual case histories showed that all of the experimental subjects were probably receiving minimal or sub-minimal amounts of vitamin A. Accumulated data indicated that this deficiency had prevailed for months in the case of the women and students and practically the entire life of the children. From the study of the diets of the children and students it was evident that the deficiency was due, in the main, to the continued use of foods low in vitamin A. Such foods as rice, grits, biscuit, corn-meal and pork made up the principal food items included in the diets, while eggs, milk, butter and green and yellow vegetables were used sparingly or only occasionally. Neither milk nor butter had been used in the regular diet of the students for more than a year and the yearly consumption of these foods by the children was limited to a few months. In the case of the women a somewhat different dietary history was found. All the women were overweight and for that reason the daily food intake was restricted to from 1000 to 1200 calories. The diets varied somewhat in detail but all included butter-milk, skim-milk, coffee, leafy vegetables, fruit, lean beef and lamb, fish and chicken. It will be noted that diets made up of foods in this list would not necessarily be low in vitamin A but because of restricted intake and possible decreased absorption and utilization, a deficiency apparently existed.

Descriptive observations. The physical examination of these experimental groups showed that the general symptoms were upon the whole similar. Individual histories revealed that in all cases loss of vigor, nervousness and general weakness were the first noticeable symptoms, while the more specific ones included dry hair and skin, brittle nails, and eye defects.

The children had lost the alertness of health and were listless and inattentive; but conjunctivitis was the most outstanding defect in this group. In a few cases the eye condition had progressed so far that there was an actual involvement of the cornea and the conjunctiva appeared slaty. Also the skin had become dry and desquamated. These flaky, desquamated areas were particularly noticeable on the back of the forearm, the upper arm near the tip of the shoulder, on the calves of the legs and across the chest. Many of the children had no subcutaneous fat so that even the skin on the face was shriveled and dry. The hair was also dry, had lost its normal gloss, and appeared bleached and lifeless.

In the women and students the external symptoms were less clearly defined. Both groups had eye defects which varied in the different subjects from minor ones such as drowsiness, burning and redness of the lids to well defined conjunctivitis. Nearly all the women, and approximately half of the students, wore glasses. Repeated checking of the glasses by reputable oculists showed that the patients were correctly fitted. But in spite of this the continued use of the eyes for several hours, as in reading or sewing, was impossible; and the condition was even more aggravated at night.

Nearly all of the adults had some skin defects. These were particularly noticeable in the women, where they appeared as desquamated areas under chin, on the sides of the neck, and across the chest. A few of the students also had dry patches on the face which became evident immediately after shaving.

DATA AND DISCUSSION. The results presented in figures 1, 2 and 3 and table 1 cover the blood pictures of 157 subjects, including 84 school children, 28 college students and 45 women.

The figures show graphically the differential leucocyte counts for these subjects before and after treatment with vitamin A. In table 1 are given the individual records of 14 women.

The blood picture of all of the subjects showed several variations from normal hematologic standards. Among these were a decrease in total leucocytes and polymorphonuclears, an increase in juveniles and large lymphocytes, and degeneration, especially of the granulocytes. Whether or not the low total leucocyte count is traceable to a deficiency of vitamin A is open to question. The number of leucocytes of normal individuals, if taken without regard to time of day or physiological conditions, which

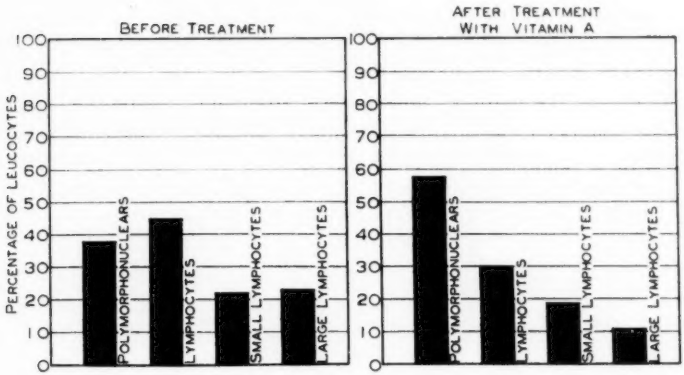

Fig. 1

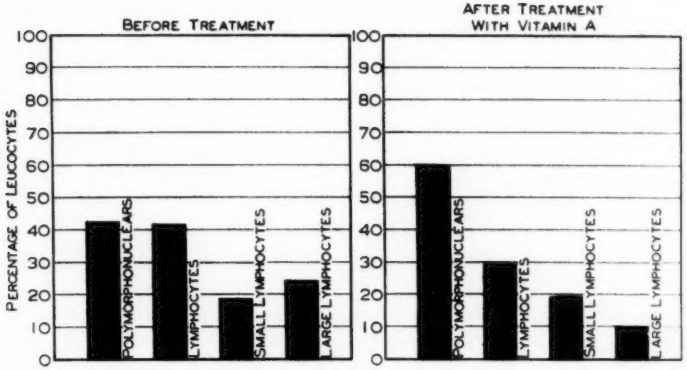

Fig. 2

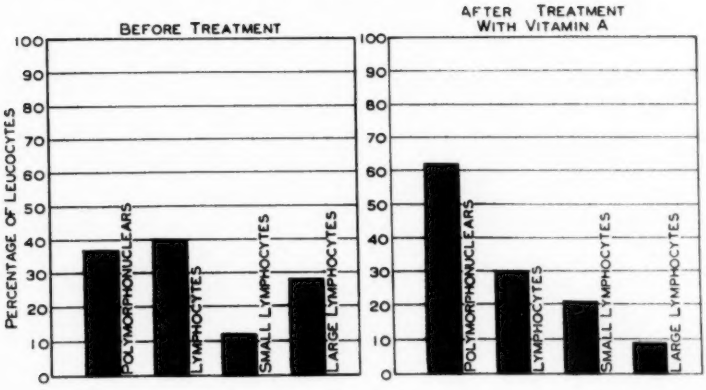

Fig. 3

TABLE 1

Blood picture of 14 women on restricted or reducing diets before and after treatment with vitamin A

CASE NO	BLOOD PICTURE BEFORE AND AFTER TREATMENT	HBG*	TOTAL LEUCOCYTES	TOTAL ERYTHROCYTES	DIFFERENTIAL LEUCOCYTE COUNT								REMARKS
					PMN	Stabs	S. Lymphs.	L. Lymphs.	L. Mono.	Juvenile	Bas.	Eos.	
		gms.			D†								
1	B.T.	15.2	6,900	4,510,000	37 (4)	5	28	22	2	4	1	1	Drastic reducing diet. Dry hair and skin. Marked malnutrition. Eye defects
	A.T.	16.0	7,500	4,700,000	66 (1)	4	17	9	1	2	1		
2	B.T.	14.3	4,000	4,325,000	44 (2)	12	6	25	2	9	1	1	Marked malnutrition. Eye defects
	A.T.	15.2	7,200	4,400,000	68 (1)	4	18	7	1	2			
3	B.T.	12.7	4,500	4,175,000	16 (12)	28	14	26	2	14			Marked malnutrition. Eye defects
	A.T.	13.9	6,500	4,495,000	68 (3)	2	20	5	1	2	1	1	
4	B.T.	11.7	5,400	3,980,000	50 (3)	10	5	20	2	12	1		Malnutrition with sinusitis
	A.T.	13.2	7,900	4,800,000	66 (2)	5	18	7	2	2			
5	B.T.	12.7	5,000	4,650,000	40 (5)	6	14	30	4	3	2	1	Reducing diet with dry hair and skin. Eye defects
	A.T.	14.9	6,800	4,725,000	62 (2)	2	22	6	3	2	2	1	
6	B.T.	12.7	5,000	4,485,000	52 (4)	8	10	20	4	2	2	2	Restricted diet. Eye defects. Dry hair and skin
	A.T.	13.8	5,500	4,625,000	64 (0)	6	18	6	3	2	1		
7	B.T.	13.8	4,800	4,110,000	52 (10)	9	11	21	1	5	1		Reducing diet. Dry hair and skin
	A.T.	14.2	5,000	4,098,000	60 (2)	4	21	5	4	3	2	1	
8	B.T.	15.8	4,300	3,900,000	38 (11)	7	11	34	3	5	1	1	Reducing diet. Ear infection
	A.T.	16.0	6,850	4,400,000	57 (3)	5	21	11	3	1	2		
9	B.T.	16.0	5,650	5,340,000	34 (8)	2	20	36	4	1	2	1	Extreme malnutrition. Inflamed eyes
	A.T.	16.0	8,350	5,500,000	51 (1)	2	26	13	5	0	2	1	
10	B.T.	12.8	4,000	4,175,000	46 (7)	4	14	26	4	3	2	1	Malnutrition. Conjunctivitis
	A.T.	13.7	5,900	4,180,000	58 (1)	2	21	11	3	2	2	1	
11	B.T.	11.8	4,500	3,975,000	42 (9)	4	21	20	3	6	2	2	Restricted diet. Throat infection
	A.T.	11.0	6,000	4,125,000	64 (2)	4	20	6	2	2	1	1	
12	B.T.	12.9	5,400	4,700,000	21 (11)	6	28	38	2	4	1		Food allergy. Dry hair and skin. Minor eye defects
	A.T.	13.1	6,200	4,816,000	58 (3)	5	24	7	2	1	2	1	
13	B.T.	13.0	5,000	4,515,000	24 (7)	6	19	35	4	9	2	1	Restricted diet. Colitis. Minor eye defects
	A.T.	13.7	6,500	4,600,000	65 (2)	3	22	8		2			
14	B.T.	12.9	4,200	3,917,000	30 (6)	4	17	37	2	7	1	2	Restricted diet. Intestinal disturbance. Dry hair, skin
	A.T.	13.7	6,100	4,125,000	55 (4)	4	27	7	2	3	1	1	

* 16 grams Hb per 100 cc. of blood = 100%.

† D — degenerate PMN.

are known to cause variation, shows a fairly wide range. However, by comparison with the leucocyte ranges for normal adults, it will be noted that in many of these cases the number of leucocytes was less than the low limit (5000 per cmm.). Even after the administration of large amounts of vitamin A the count was still in the low range.

Normal blood contains from 65 to 70 per cent of adult segmented neutrophiles; but in this work it was found that these cells make up only 16 to 52 per cent of the total count. The remainder are younger forms as stabs, juveniles, and myelocytes. These young cells sometimes constituted as much as 30 to 42 per cent of the total count. The presence of this unusual number of immature circulating neutrophiles indicated that the bone marrow was being damaged.

In the study of the blood picture of rats on a vitamin A deficient diet, Abbott and Ahmann (1) determined the condition of the marrow in the long bones. The usual finding was a gelatinous degeneration of the marrow, but occasionally an almost complete replacement by fibrous stroma had occurred. It is possible that a similar condition exists in humans suffering from a severe deficiency of vitamin A.

The normal hematologic standards for adults show that the circulating lymphocytes make up from 20 to 35 per cent of the total differential count, while in children the percentage is somewhat higher. About 75 per cent of the lymphocytes are small, the remainder large. But in this study of individuals on a diet low in vitamin A it was shown that the large lymphocytes make up the larger part of the lymphocyte count.

In many cases the small lymphocyte was rarely seen and the typical cell was the large or intermediate form. It is generally agreed that large lymphocytes are the more immature. As a rule it may be assumed that the presence of large lymphocytes in abnormal numbers indicates lymphoid hyperplasia. In this respect the immaturity of the lymphocytes is similar to the granulocytic immaturity, both of which were constant findings in this study.

Degeneration, often occurring in the leucocytes, was most noticeable in the polymorphonuclears. These cells often showed toxic granules and vacuolated cytoplasm, and if the deficiency had progressed to an advanced stage the cytoplasm appeared as faintly stained strands, the nucleus fragmented and granular and the cell wall indistinct and broken. The lymphocytes were also abnormal as to size and shape, and bizarre forms were not uncommon.

With the daily administration of 51,000 U.S.P. units of vitamin A over a period of 2 to 4 weeks a gradual improvement in the general condition of all the subjects was noted and in about 6 weeks the ratio of polymorphonuclears to lymphocytes returned to normal and the other abnormalities gradually disappeared. However, in several cases an unusually high

percentage of large lymphocytes persisted even after the disappearance of the eye defects, and dry hair and skin.

In this investigation a study has been made of the differential leucocyte counts of 157 individuals whose diets and symptoms indicated a vitamin A deficiency. The characteristic changes which were constant in the blood picture of these subjects are as follows: 1, a mild leucopenia; 2, a decrease in the polymorphonuclear neutrophils; 3, a relative increase in large lymphocytes with a corresponding drop in small lymphocytes; 4, the occurrence of degenerate cells, and 5, an increase in the number of juveniles. It will be noted that this blood picture is similar to that of rats fed a diet deficient in vitamin A and, as in the case of the rats, the administration of large amounts of vitamin A brought about a gradual improvement in symptoms and in a few weeks the differential count was within the normal range.

From this work it was concluded that the differential leucocyte count is of value in diagnosing a deficiency of vitamin A in man.

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THE ACTION OF LEAD ON PHOSPHOCREATINE IN THE MUSCULAR PARALYSIS OF LEAD POISONING

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The exact mechanism of lead palsy is still uncertain. In a review by Aub, Fairhall, Minot and Reznikoff (1926) it is noted that almost all the investigations of lead palsy have been of a pathological and clinical nature, and that very little direct experimental work has been done. Clinically, the primary cause of the paralysis is generally considered to be in the motor nerve. Nevertheless, a few early investigators (Gusserow, 1861; Harnack, 1878; Mellon, 1913) have obtained experimental evidence indicating that lead produces a definite physiological lesion in skeletal muscle. These workers found that muscles poisoned with lead rapidly became fatigued and inexcitable. Since this effect occurred on direct stimulation, the contractile elements themselves appeared to be affected. Reznikoff and Aub (1927), moreover, in one instance obtained action potentials from the nerve but not from the muscle of a "leaded" nerve-muscle preparation. They also observed that the action current from a poisoned muscle diminished rapidly after fatigue and soon disappeared, while that of the control muscle was unaffected. Furthermore, they found no difference in the action currents obtained from normal and "leaded" nerves. From these experiments they concluded that lead has no deleterious action on nerve, while it has a definite injurious effect on muscle. These results cannot, however, be considered conclusive, because of the limited number of experiments and the technical difficulties encountered.

Reznikoff and Aub (1927) isolated both gastrocnemii from a frog, and after the diffusion of inorganic phosphate became constant put one muscle into Ringer containing lead chloride (0.05 mgm. Pb per cc.). At the end of one hour they found that the average rate of diffusion of inorganic phosphate had increased 320 per cent, while the average rate of diffusion from the control muscle had decreased 10 per cent during the same period. Since this work was just previous to the discovery of phosphocreatine, the authors had proceeded on the basis of the then-prevailing view; namely, that most of the acid-soluble phosphate in the muscle is present as in-

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organic phosphate. Hence, they concluded that lead alters the permeability of the muscle cell-membrane.

With the discovery of phosphocreatine in 1927 by Fiske and Subbarow, and by Eggleton and Eggleton (phosphagen), it was found that only 15 to 20 per cent of the total acid-soluble phosphorus of the muscle is present as true inorganic phosphate. Lundsgaard (1930a), working with muscles poisoned with monoiodoacetic acid, has shown that the breakdown of phosphocreatine supplies energy for muscular contraction. Phosphocreatine, being a highly unstable organic compound, is easily hydrolyzed into inorganic phosphate and creatine. Eggleton (1930) has stated that creatine and the phosphate ion are freely diffusible through the muscle membrane, whereas phosphocreatine cannot so diffuse. Consequently, since we know now that the true inorganic phosphate content of muscle is low (20 to 25 mgm. per cent), the following question arises: Might not the large increase in the rate of diffusion of phosphate from "leaded" muscles (Reznikoff and Aub, 1927) be due to hydrolysis of phosphocreatine thus increasing the concentration of inorganic phosphate in the muscle? In the light of our newer knowledge of the chemical events of muscular contraction, it appears that this and other questions concerning the action of lead on skeletal muscle would be best answered by an experimental study of the phosphocreatine metabolism of muscles from lead-poisoned animals.

METHOD. Frogs were poisoned with lead by keeping them in an approximately 0.01- to 0.02-per-cent lead-chloride solution, changed daily. These animals were used for experimentation after varying intervals of exposure. At the end of 12 to 14 days, the characteristic symptoms of poisoning appeared: the frogs had lost weight, were sluggish and moved about with difficulty. In another series, the animals were exercised daily for a few minutes by repeated prodding. This was continued until the animal showed signs of fatigue. In this series the symptoms of poisoning appeared sooner (2 to 5 days). No animal was exercised for at least 24 hours before it was used for an experiment. The work was done during the summer months, and it was found that the best results were obtained when the frogs were kept at a low temperature. Accordingly, a day before the experiment, the animal was placed in a cold-room (3 to 8°C.) where all further procedures were carried out. Nembutal (0.5 mgm. per 10 grams body weight) injected into the dorsal lymph sac was used for anesthesia. In later experiments some of the animals were pithed. The sciatic nerve of one leg was exposed, and stimulated with a maximal tetanizing current for two minutes. The opposite, unstimulated gastrocnemius served as a control. At the end of varying periods of recovery the stimulated gastrocnemius and the control were carefully dissected out and immediately frozen in liquid air. In some cases the control

muscle was removed before stimulating the other. A corresponding series of experiments on normal frogs was also made. The muscles were analyzed for phosphocreatine, inorganic phosphate and total phosphate, using the methods described by Fiske and Subbarow (1925, 1929). For determination of phosphocreatine and inorganic phosphate, the calcium-precipitation method was chosen in preference to the direct colorimetric method, because of the high room-temperature. The preparation of the trichloracetic-acid filtrate and the addition of the calcium-chloride solution were done in the cold-room. By this method the phosphocreatine is determined directly.

Normal. In 47 experiments the average value of phosphocreatine found in normal unstimulated muscle was 54.6 ±0.5 mgm. per cent, while the

TABLE 1

Normal animals

Values expressed as milligrams per cent of P

	NUMBER OF EXPERIMENTS	PHOSPHOCREATINE		INORGANIC PHOSPHATE		SUM		TOTAL PHOSPHATE		RESIDUAL PHOSPHATE	
		S	C	S	C	S	C	S	C	S	C
A. No recovery	8	27.2 ±1.9	56.2 ±0.85	55.6 ±2.2	28.6 ±1.6	82.8 ±1.8	84.7 ±1.3	150.3 ±1.2	143.2 ±0.97	67.5 ±1.8	58.5 ±1.3
B. Recovery 1 min.	9	45.2 ±1.1	54.3 ±0.66	39.5 ±1.1	33.1 ±0.6	84.7 ±0.89	87.4 ±1.0	140.8 ±1.8	140.3 ±2.0	56.1 ±1.7	52.9 ±1.3
C. Recovery 2 min.	17	45.7 ±1.0	54.9 ±1.0	42.4 ±1.0	33.6 ±0.6	88.1 ±1.1	88.4 ±2.0	147.7 ±1.2	148.0 ±1.1	59.6 ±0.81	59.6 ±0.75
D. Recovery 30 min.	13	52.9 ±1.3	53.8 ±1.0	33.5 ±1.3	34.1 ±1.1	86.4 ±1.2	88.3 ±1.1	146.4 ±1.4	147.0 ±0.88	60.1 ±1.1	58.7 ±1.0
Averages			54.6 ±0.5		32.7 ±0.5		87.0 ±0.6		145.4 ±0.7		57.8 ±0.6

S = stimulated muscle. C = control muscle.

inorganic phosphate was 32.7 ±0.5 mgm. per cent. These figures are in agreement with those obtained by other workers on frog muscle (Eggleton and Eggleton, 1927; Lundsgaard, 1930a).

If a muscle is stimulated for 2 minutes and immediately frozen, about 50 per cent of the phosphocreatine is hydrolyzed (table 1, A). If a recovery period of 1 minute is allowed, about 60 per cent of the phosphocreatine hydrolyzed is resynthesized (table 1, B). Nachmansohn (1928) and Lundsgaard (1934) have found that 30 per cent of the phosphocreatine hydrolyzed during a tetanus is anaerobically resynthesized and that this resynthesis occurs during the first 20 or 30 seconds after the end of the tetanus. Consequently, the rate of resynthesis of phosphocreatine is

quite rapid during the first minute, and was found here to average 18 mgm. per cent. Sacks and Sacks (1935) reported that the average rate in the cat was 16 mgm. per cent. Meyerhof and Nachmansohn (1930) found that the oxidative resynthesis of phosphocreatine is much slower in frog muscle than is the anaerobic resynthesis. Accordingly, during the second minute of recovery very little phosphocreatine is resynthesized (table 1, C). *However, at the end of a half-hour recovery, the phosphocreatine hydrolyzed during a two-minute tetanus is completely resynthesized* (table 1, D).

TABLE 2

Lead-poisoned animals

Values expressed as milligrams per cent of P

	NUMBER OF EXPERIMENTS	PHOSPHOCREATINE		INORGANIC PHOSPHATE		SUM		TOTAL PHOSPHATE		RESIDUAL PHOSPHATE	
		S	C	S	C	S	C	S	C	S	C
A. (2–20 days) Recov. 30′	24	29.9 ±1.4	37.1 ±1.6	47.5 ±1.7	43.1 ±2.0	77.5 ±1.6	80.3 ±1.4	143.5 ±1.8	140.8 ±1.8	66.1 ±1.2	63.1 ±1.3
B. (2–6 days) Recov. 30′	9	32.0 ±2.7	37.6 ±2.9	47.4 ±2.9	40.8 ±2.7	79.4 ±1.4	78.4 ±1.4	146.0 ±1.2	147.8 ±1.5	66.6 ±1.2	69.4 ±1.4
Exercised											
C. (2–5 days) No recov.	8	10.1 ±1.6		65.7 ±2.9		75.8 ±2.9		141.3 ±4.4		65.5 ±2.4	
D. (2–5 days) Recov. 30′	14	15.3 ±1.5	24.2 ±2.9	58.7 ±2.3	52.4 ±3.0	74.0 ±1.1	76.6 ±1.4	135.9 ±2.0	137.1 ±1.8	61.9 ±1.1	60.5 ±1.3
E. (10–20 days) Recov. 30′	10	10.6 ±1.0	20.1 ±0.76	42.9 ±1.3	36.6 ±1.8	53.5 ±1.8	56.7 ±1.8	110.3 ±2.6	109.3 ±2.8	56.8 ±2.0	52.7 ±1.7

S = stimulated muscle. C = control muscle.

Lead-poisoned. From the results of the individual experiments on the non-exercised series it appeared that there might be a correlation between the duration of exposure to lead and the changes in phosphocreatine. Yet a statistical analysis did not reveal any marked differences between one group exposed from 2 to 6 days and another poisoned from 9 to 12 days. After 15 days' exposure, however, the loss of phosphocreatine was somewhat more than that found in the animals poisoned from 1 to 10 days; but since the former group was relatively small (6 experiments) it was included with the others. Thus the averages recorded in table 2, A, have been obtained from 24 animals exposed to lead from 2 to 20 days.

The resting level of phosphocreatine (37.1 ±1.6 mgm. per cent) found

in this series of lead-poisoned animals is lower than that of normal frogs, while the inorganic phosphate, on the other hand, is increased (43.1 ±2.0 mgm. per cent). Moreover, it can be seen from table 2, B, that these changes take place in animals exposed to lead for a relatively short time (2 to 6 days).

The effect of exercise in the series exposed from 2 to 5 days (table 2, D) was to cause a further lowering of the resting phosphocreatine level and a greater increase in the amount of inorganic phosphate. Here, also, the phosphocreatine hydrolyzed during stimulation is not resynthesized after a half-hour recovery period.

The sum of phosphocreatine and inorganic phosphate is practically the same in both gastrocnemii. Also, it can be seen from table 1 that there is little variation in the sum of the two among the normal groups. However, if we compare the sums of the latter with those recorded in the poisoned series A, B, C, and D (table 2), it is apparent that the sum of phosphocreatine and inorganic phosphate is somewhat lower in the leaded groups. There appears, therefore, to be a loss of inorganic phosphate in the lead-poisoned animals; but, presumably because the duration of exposure of the exercised group was short, the loss is not great. This loss is likewise seen in the non-exercised series. After a few days' poisoning the animals are not active and hardly move about. For that reason, apparently, the changes seen after 20 days are not much greater than after 6 days' exposure (supra). Accordingly, it seemed probable that if a group of animals were exercised daily and poisoned for a longer interval there would be an opportunity for a greater loss of inorganic phosphate. The results of the study of such a series are recorded in table 2, E. Not only is there a further lowering of the phosphocreatine level, but also a loss of inorganic phosphate; so that the sum of the two is now decidedly less than that of the other poisoned series.

Diffusion of phosphate from normal and "leaded" isolated muscle. Reznikoff and Aub (1927) found an increase in the diffusion of inorganic phosphate from a muscle immersed in lead-Ringer. Since they did not analyze the muscle, it appeared of interest to repeat their experiments and, in addition, analyze the muscle for phosphocreatine, inorganic phosphate, and total phosphate. The method was as follows: both gastrocnemii were carefully dissected out from the pithed frog. One was put in a beaker containing 15 cc. Ringer (NaCl, 0.6 per cent; KCl, 0.03 per cent; $CaCl_2$, 0.02 per cent), while the other was immersed in a similar volume of Ringer containing $PbCl_2$ (0.05 mgm. of Pb per cc.). Both solutions were continuously oxygenated. At the end of one hour, the muscles were frozen in liquid air and analyzed. The Ringer solutions were also separately analyzed for inorganic phosphate. The results are recorded in table 4.

The only modification of the method of Reznikoff and Aub was that the muscles were not placed first in normal Ringer until the diffusion of phosphate became constant. However, since the diffusion of phosphate from the control muscles during the hour is practically the same in all the

TABLE 3

Values expressed in milligrams per cent of P

NORMAL ANIMALS		"LEADED," EXERCISE ANIMALS	
Phosphocreatine—resting muscle	56.2 ±0.85	Phosphocreatine—control muscles	24.2 ±2.9
Phosphocreatine—after 2 min. stimulation	27.2 ±1.9	Phosphocreatine—after 2 min. stimulation	10.1 ±1.6
Phosphocreatine hydrolyzed after 2 min. stimulation	29.0 ±2.0	Phosphocreatine hydrolyzed after 2 min. stimulation	14.1 ±3.3
Phosphocreatine—after 30 min. recovery	52.9 ±1.3	Phosphocreatine—after 30 min. recovery	15.3 ±1.5
Phosphocreatine—after 2 min. stimulation	27.2 ±1.9	Phosphocreatine—after 2 min. stimulation	10.1 ±1.6
Phosphocreatine resynthesized after 30 min. recovery	25.7 ±2.3	Phosphocreatine resynthesized after 30 min. recovery	5.2 ±2.2
Phosphocreatine—resting normal muscle	53.8 ±1.0	Phosphocreatine—resynthesized in normal muscle	25.7 ±2.3
Phosphocreatine—resting "leaded" muscle	37.1 ±1.6	Phosphocreatine — resynthesized in "leaded" muscle	5.2 ±2.2
Difference	16.7 ±1.9	Difference	20.5 ±2.5

TABLE 4

Isolated muscles

Control in normal oxygenated Ringer; other muscle in Pb-oxygenated Ringer
Values expressed in mgm. per cent of P

PHOSPHOCREATINE		INORGANIC PHOSPHATE		DIFFUSION OF PHOSPHATE INTO RINGER		SUM OF INORGANIC PHOSPHATE + PHOSPHOCREATINE		TOTAL PHOSPHATE IN MUSCLE	
Muscle in Pb Ringer	Control	Pb	Control	Pb	Control	Pb	Control	Pb	Control
24.2	33.6	66.3	59.1	6.8	4.9	90.5	92.7	158.1	153.2
23.7	38.0	60.7	49.8	5.2	3.4	84.4	87.8	147.9	149.2
20.6	34.1	60.3	50.7	4.3	2.7	80.9	84.8	137.6	146.9
28.9	45.6	54.7	46.8	9.2	2.9	83.6	92.4	140.0	153.0
31.7	41.9	45.7	37.8	4.1	3.2	77.4	79.7	140.2	139.4
22.6	35.8	57.9	45.5	4.7	3.2	80.5	81.3	145.4	141.5
27.4	38.8	52.5	46.3	6.5	3.3	79.9	85.1	140.8	143.7

experiments, it did not appear necessary to carry out the above procedure. In all cases, the diffusion of inorganic phosphate was greater from the muscles kept in the Pb-Ringer. Furthermore, the inorganic phosphate of the "leaded" muscle was higher, while the phosphocreatine was correspondingly lower than in the control muscle.

DISCUSSION AND CONCLUSIONS. In the normal animal the phosphocreatine hydrolyzed during a two-minute stimulation period is completely resynthesized after a half-hour recovery. The lead-poisoned animals, on the other hand, not only have a much lower resting level of phosphocreatine, but there is very little resynthesis, if any, of phosphocreatine in the stimulated muscles during the half-hour recovery period. A comparison between the normal and the exercised poisoned series shows that about half as much phosphocreatine is hydrolyzed during stimulation of the latter (table 3). In the same table we can also see that the average amount of phosphocreatine resynthesized by the poisoned muscles is 5.2 $\pm$2.2 mgm. per cent. If we accept the criterion for the significance of such a number, that it be greater than three times its probable error, then this figure is not significant. Thus, the figures do not prove that any resynthesis of phosphocreatine occurs within the half-hour.

Normally, when phosphocreatine breaks down, the inorganic phosphate increases correspondingly, so that the sum of the two remains the same. In the exercised poisoned animals the phosphocreatine level is initially low, and, although the inorganic phosphate is greatly increased, the sum tends to be less than normal. As the duration of poisoning is lengthened, the loss of phosphocreatine and inorganic phosphate becomes greater; and the sum is now much less than normal (table 2, E). The large increase in inorganic phosphate is undoubtedly due to hydrolysis of phosphocreatine and its failure to be readily resynthesized.

The effect of exercise is strikingly brought out by comparing the results of the two groups exposed to lead for the same length of time, but with only one group exercised (table 2, B and D). The changes in phosphocreatine and inorganic phosphate are much greater in the latter group.

The difference between the total phosphate and the sum of phosphocreatine and inorganic phosphate is the value for the non-phosphocreatine organic phosphate. This fraction includes hexosephosphate and adenosine triphosphate, and is designated in tables 1 and 2 as residual phosphate. The average resting value of the residual phosphate in the normal is 57.8 $\pm$0.6 mgm. per cent, but in the lead series A, B, C, and D (table 2) it is somewhat higher. From the data here presented we cannot tell what changes have taken place in the components of this fraction. Nevertheless, in table 2, E, we see that although there has been a loss of phosphocreatine and inorganic phosphate, the residual phosphate is now within the normal range. It will be necessary, however, to study the changes in hexosephosphate and adenosine triphosphate to account for the initial increase and later decrease in the residual phosphate of lead-poisoned muscles.

The results of the diffusion of inorganic phosphate from isolated muscles immersed in normal and Pb-Ringer agree with those of Reznikoff and Aub (1927). Because they believed that most of the acid-soluble phosphate in

the muscle was in inorganic form, they concluded that the mode of action of lead was to alter the permeability of the muscle cell. Stella (1928), however, found that inorganic phosphate was freely diffusible and that the diffusion constant was the same for resting and fatigued muscle. Furthermore, he concluded that there was no evidence for any change in the permeability to phosphate of the muscle fibers. "The fact that phosphate diffuses more rapidly from a fatigued muscle, and far more rapidly from a muscle in rigor, is due simply to the greater concentration of phosphate in these two cases." Analysis of the muscle has shown that the phosphocreatine level in the "leaded" muscle is low while the inorganic phosphate is increased. In view of Stella's results, the diffusion of inorganic phosphate is dependent on the ratio of phosphocreatine to inorganic phosphate in the muscle. Since in all the experiments the phosphocreatine level of the "leaded" muscle is lower than its corresponding control, lead apparently can hydrolyze phosphocreatine. Consequently, the increase in inorganic phosphate establishes a greater diffusion gradient. If the permeability of the cell is also altered, it probably acts conjointly with the above factor to produce an increased diffusion of inorganic phosphate from a "leaded" muscle.

Phosphocreatine plays a fundamental and significant part in the series of chemical events that occur during muscular contraction. Fiske and Subbarow (1929) found a direct correlation between the presence of phosphocreatine and the ability of a muscle to contract. Lundsgaard (1930) also reported that the tension developed in muscles poisoned with monoiodoacetic acid is proportional to the breakdown of phosphocreatine. For the maintenance of contraction it is necessary that the phosphocreatine hydrolyzed be rapidly resynthesized, if the complex cycle of chemical reactions is to be repeated. If, however, the phosphocreatine is not restored, an important link in the chain of events is broken and the muscle cannot further contract.

The results of the present experiments show that lead causes a marked loss of phosphocreatine from skeletal muscle. Since the amount of phosphocreatine hydrolyzed during stimulation is much less than that normally hydrolyzed, the poisoned muscle cannot develop and maintain any appreciable tension; and because the phosphocreatine is not readily resynthesized, the muscle soon becomes inexcitable. Thus the low level of phosphocreatine and the marked interference with its resynthesis can readily explain the general muscular weakness, ready fatigability and palsy resulting from exposure to lead.

SUMMARY

Phosphate changes were studied in muscles of normal and lead-poisoned frogs. Normally, the phosphocreatine hydrolyzed during a two-minute tetanus was completely resynthesized after a half-hour recovery period.

The resting level of phosphocreatine in the muscles of poisoned animals was much lower than the normal value, while the inorganic phosphate was greatly increased.

At the end of a half-hour recovery, there was practically no resynthesis of the phosphocreatine hydrolyzed during a two-minute tetanus of the lead-poisoned muscle.

There is a loss of inorganic phosphate from the poisoned muscle due to the increase in inorganic phosphate content of the muscle as a result of the hydrolysis of phosphocreatine.

Muscles immersed in oxygenated Ringer containing lead have a lower phosphocreatine value and a higher inorganic phosphate content than control muscles. The increase in the rate of diffusion of phosphate from a muscle in Pb-Ringer is explained by the increase in inorganic phosphate which sets up a greater diffusion gradient.

On the basis of our present views concerning the chemistry of muscular contraction, the results can explain the general muscular weakness, ready fatigability and palsy resulting from exposure to lead.

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THE EFFECT OF DUODENAL INSTILLATION OF HYDROCHLORIC ACID UPON THE FASTING BLOOD SUGAR OF DOGS

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It has been repeatedly reported that extracts possessing hypoglycemic properties may be prepared from the mucosa of the upper intestine (1–21). It has been proposed that the active constituent of these extracts is a hormone which is concerned with the regulation of carbohydrate metabolism. In order to provide some measure of support for this hypothesis attempts have been made to demonstrate that the presence of HCl in the duodenum lowers the blood-sugar level, just as it stimulates the external secretion of the pancreas (19, 25–34). This evidence is not entirely convincing and furthermore certain investigators have been unable to show that the presence of acid in the duodenum exerts any effect on the fasting blood-sugar level (29, 32–34). Accordingly, we have reinvestigated the problem by studying the effects of intraduodenal administration of HCl on the fasting blood-sugar level in dogs. In view of the fact that our experimental results are contradictory to most of the evidence which has been advanced in support of the hypothesis that the duodenum exerts an endocrine control of carbohydrate metabolism, we have deemed it advisable to analyze the experiments of previous investigators.

EXPERIMENTAL. All animals employed in these experiments were subjected to a preliminary fast of approximately 18 hours. Somogyi's zinc hydroxide blood filtrates (22) representing 2.0 cc. of venous blood were employed in the duplicate determination of blood-sugar by the method of Schaffer, Hartman, and Somogyi (reagent no. 2) (23). The maximum error of the method, $\pm$ 3.7 mgm. per cent, was determined by making duplicate analyses of 20 aliquot portions of the same blood sample.

In the majority of our experiments 0.3 per cent HCl in 50 to 100 cc. quantities have been employed. The duodenal mucosa is sensitive to acid (24) and is never, under normal conditions, subjected to the influence of the large amounts of 0.5 to 0.8 per cent acid which some investigators

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have used. The experiments have been controlled by the substitution of 0.9 per cent NaCl for the dilute acid.

RESULTS. *Experiments upon unanesthetized dogs.* The fasting blood-sugar concentration of unanesthetized dogs following intestinal stimulation with HCl has been determined in 3 groups of animals. Five experiments were made upon 2 completely gastrectomized dogs with jejuno-esophageal anastomoses. Single experiments were made upon 4 dogs with duodeno-esophageal anastomoses and pouches of the entire stomach. A small rubber tube was introduced by mouth and passed into the esophagus permitting the introduction of acid directly into the duodenum and jejunum at a slow rate during a one-hour period. The administration of acid to the third group, the normal intact dogs, was carried out in the same manner as described above except that the acid was introduced into the stomach in approximately 5 minutes. A total of 9 experiments were performed upon 4 dogs. The animals were all in good physical condition and trained to stand in stocks for long periods of time. Each received 100 cc. of 0.3 per cent HCl.

Inspection of the data secured from unanesthetized animals (table 1, group A) reveals that the fasting blood-sugar concentration was not altered during the five-hour interval following the introduction of acid into the intestine, despite the fact that in the animals with intestinal-esophageal anastomoses the acid came into direct contact with the intestinal mucosa for at least a period of one hour.

Our experiments upon gastrectomized and total pouch dogs are comparable to those made by Baisset (35) except that we did not employ such large quantities of acid, and he failed to control his experiments by substituting water or saline for the acid. The slight hypoglycemic responses (8–15 mgm. per cent) which he observed were attributed to the acidosis (see also ref. 37) caused by the secretion of alkaline pancreatic juice.

Of the eight previous publications (28, 29, 32–37) pertaining to the subject under discussion, only two (28, 35) report that the introduction of dilute HCl into the duodenum or stomach causes any lowering of the fasting blood-sugar level in unanesthetized dogs, rabbits, or humans. Since no control experiments were performed in these two cases our own negative results confirm the conclusion that the presence of HCl in the duodenum does not lower the fasting blood-sugar level in unanesthetized animals.

Intact anesthetized (pentobarbital) dogs. Since most of the experiments dealing with the blood-sugar level have been performed on anesthetized animals, we conducted a series of experiments on dogs under pentobarbital (nembutal) anesthesia. Following laparotomy, a hypodermic needle was inserted into the lumen of the duodenum and attached to rubber tubing. Solutions at body temperature could thus be injected into the duodenum

without disturbing the animal. Blood samples were taken for a control period of two hours after operative intervention. Then, eight test animals received an intraduodenal injection of 0.3 per cent HCl; 4 animals received 0.5 per cent HCl. The same quantity (50 cc.) of 0.9 per cent saline was injected into the duodenum of the 8 control animals. The solutions were introduced slowly over a 20-minute period.

The average blood-sugar concentration of the control group of animals receiving saline and of those receiving 0.3 and 0.5 per cent HCl via the

TABLE 1

Blood-sugar values of fasting unanesthetized and anesthetized dogs before and after introducing dilute HCl into the intestine or stomach

The data given are averages

PROCEDURE AND TYPE OF ANIMAL PREPARATION	NO. OF DOGS	NO. OF TESTS	BLOOD-SUGAR CONCENTRATION (MGM. %)							
			Control period		After introduction of 100 cc. 0.3% HCl during first hour					
			0 hr.	0.5 hr.	0.5 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
Group A (unanesthetized):										
Gastrectomized, jejuno-esophageal anastomosis	2	5	69	71	70	73	69	71	71	73
Total pouch, duodeno-esophageal anastomosis	4	4	76	76	76	78	76	74	76	76
Intact (acid in stomach)	4	9	78	82	83*	82	82	79	79	80
			Control period		Dilute acid or saline in duodenum during first 20 minutes					
			1.5 hr.	2 hr.	0.5 hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
Group B (anesthetized):										
50 cc. 0.9% NaCl in duodenum	8	8	93	93	90	91	87	90	90	90
50 cc. 0.3% HCl in duodenum	8	8	81	82	81	84	84	82	86	85
50 cc. 0.5% HCl in duodenum	4	4	81	82	82	82	82	81	81	82

* Acid introduced into the stomach during a five-minute period.

The dogs weighed from 8 to 25 kilos, the average being 12 kilos.

duodenum was not appreciably altered during a five-hour period (table 2, group B).

Freud and Saadi-Nazim (25) introduced dilute HCl into the duodenum of 7 dogs under chloralose anesthesia, which had an initial blood-sugar of 170 mgm. per cent and one hour later of 140 mgm. per cent. Such high blood-sugar levels cannot be regarded as fasting values, and since no control experiments were performed, these results are of doubtful significance. Baisset (35), who performed similar experiments, obtained a 5 to 15 mgm. per cent reduction in blood-sugar and attributed his results to changes in

acid-base balance rather than to the liberation of a duodenal hormone. Zunz and La Barre (30) have stated that the intraduodenal injection of HCl in anesthetized but otherwise *intact* animals is only very rarely followed by a slight fall in blood-sugar below the initial level. Our results on *intact*, anesthetized dogs confirm the findings of Zunz and La Barre.

TABLE 2

Blood-sugar concentration of adrenal inactivated dogs after introduction of acid or saline into the duodenum

DOG NO.	WEIGHT	BLOOD-SUGAR CONCENTRATION (MGM. %) AFTER LIGATION OF ADRENAL VEINS									
		After anes.	Control period			50 cc. 0.3% HCl in duodenum during 1st 20 minutes					
			½ hr.	1 hr.	2 hr.	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
	kgm.										
1	8.2	80	106	91	83	82	80	78	79	88	95
2	7.7	94	99	96	93	88	79	75	75	74	72
3	8.2	96	96	91	83	83	79	80	84	83	84
4	8.2	80	83	77	76	69	64		70	75	81
5	17.3	78	99	90	86	86	85	89	93	92	94
6	5.9	104	101	85	69	64	60	60	58	63	64
7	13.6	81	85	85	74	72	65	66	67	65	61
8	9.1	66	73	65	60	56	49	44	51	57	64
Average....		85	92	86	78	75	70	70	73	74	77
						50 cc. 0.9% saline into duodenum during 1st 20 minutes					
1	7.7	111	122	105	94	89	79	85	99	98	109
2	12.7	81	92	90	85	85	84	86	89	92	91
3	7.3	100	102	106	96	95	97	94	107	107	116
4	7.7	95	107	95	87	87		85	80	79	77
5	13.6	103	102	103	91	91	90	91	94	94	96
6	12.3	107	115	116	113	114	110	110	113	120	119
7	15.5	84	100	87	71	69	64	75	75	75	72
8	16.1	83	86	83	82	81	80	80	80	75	73
9	12.3	96	90	85	86	83	84	83	85	81	85
10	11.8	73	75	72		56	54	47	58	67	71
Average....		93	99	94	89	85	82	83	88	89	91

Inactivation of the adrenal glands. Although the physiological significance of a mechanism for regulating carbohydrate metabolism which operates only in the absence of the adrenal glands is highly questionable, a series of experiments has been performed in animals with inactivated adrenal glands in an attempt to confirm the report of Zunz and La Barre (30), who obtained hypoglycemia in decapsulated animals on the intraduodenal instillation of acid.

Under pentobarbital anesthesia, the adrenal glands were inactivated either by ligating the veins medially and laterally to each gland or by complete bilateral extirpation. The operative procedures and manipulation of the adenal glands *frequently elevated the blood-sugar level,* so that a two-hour period was permitted to intervene before duodenal administration of acid or saline was instituted.

Eight animals with ligated adrenal veins received 50 cc. of 0.3 per cent HCl; 10 other animals received the same quantity of 0.9 per cent saline in the duodenum over a 20-minute period (table 2). Saline was introduced into the duodenum of 7 animals after extirpation of the adrenals (table 3).

A significant fact to be noted in these experiments is that there is a progressive decrease in blood-sugar during the two-hour period following

TABLE 3

Blood-sugar values of adrenalectomized dogs after introduction of saline into the duodenum

DOG NO.	WEIGHT	BLOOD-SUGAR CONCENTRATION (MGM. %) AFTER ABLATION OF THE ADRENAL GLANDS									
		After anes.	Control period				50 cc. 0.9% saline in duodenum				
			0 hr.	1 hr.	1½ hr.	2 hr.	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.
	kgm.										
1	9.7	66	80	66	67	64	57	53	69		
2	12.3		71	63	59	60	57	56	48	54	53
3	10.5	69	118	85	74	68	67	63	60		
4	10.0	86	147	92	85	84	87	105	106	74	69
5	9.0	72	122	77	84	74	64	57	55	57	73
6	7.2	103		93	89	85	88	91	83	75	
7	13.6	111	156	109	105			90			
Average		84	115	98	80	72	70	73	66	65	65

the ligation of the adrenal veins *before* acid or saline was injected into the duodenum. This decrease was not accelerated by the introduction of acid into the duodenum as a comparison with the control group indicates (table 2). Although the decrease in blood-sugar concentration varied in individual animals, the average fall in the animals receiving acid as well as those receiving saline was approximately 8 mgm. per cent in 1 to 2 hours. At the end of 5 hours the average blood-sugar concentration was restored to the pre-injection level, but not to the level which prevailed immediately after anesthetization.

Bilateral extirpation of the adrenal glands caused the average blood-sugar concentration to become elevated to from 84 to 115 mgm. per cent and then to decrease markedly to 72 mgm. per cent during the next 2

hours (table 3). Saline was introduced into the duodenum at the end of two hours but the animals usually succumbed 5 to 7 hours after adrenal ablation, the average blood-sugar concentration near the time of death being 70 mgm. per cent (6 dogs). Experimentation upon this type of animal preparation was therefore discontinued.

Gley and Hazard (27) comment upon the fact that extirpation of the adrenals leads to hypoglycemia in dogs. Their variable data from 4 animals receiving acid in the duodenum and only one control animal do not warrant the conclusion that the decrease in blood-sugar was augmented by the presence of acid in the duodenum. Zunz and La Barre (30) attributed their failure to obtain hypoglycemia in anesthetized dogs to a compensatory liberation of adrenalin by the adrenal medulla. Accordingly, they introduced 0.5 to 0.8 per cent HCl into the duodenum of chloralosed dogs one-half hour after adrenal-inactivation and determined the alteration in blood-sugar level for approximately 4 hours thereafter. According to our analysis, these authors (30, 31) have presented 13 experiments on chloralosed adrenal-inactivated dogs in which the average reduction in blood-sugar amounted to 21 per cent. These experiments were controlled by only two experiments. An average decrease in blood-sugar nearly identical in magnitude (17 per cent) occurred in our 10 control animals during the 3 hour period following ligation of the adrenal veins. Five of these 10 animals, however, showed no significant hypoglycemia. Other workers (38) have previously made the observation that one-stage bilateral adrenalectomy in dogs produces varying degrees of hypoglycemia in most but not all animals.

Discussion. Our results obtained from 64 experiments on 55 dogs have failed to yield evidence which suggests that acid in contact with the duodenum causes the production of a hormone that decreases the fasting blood-sugar level. In regard to the necessity of adrenal-inactivation, we would have reached the same conclusion as Zunz and La Barre (30, 31) if we had not performed a number of saline-control experiments, and if we had introduced dilute acid into the duodenum only one-half hour after adrenal-inactivation. In view of our results it is unnecessary to search for a duodenal hormone which contributes to the regulation of carbohydrate metabolism and whose existence can only be demonstrated when the adrenals are inactivated.

It should be pointed out, however, that our results do not disprove the existence of a duodenal hormone which regulates carbohydrate metabolism. It remains to be demonstrated that substances other than HCl are or are not active when introduced into the duodenum. Furthermore, it remains to be shown that duodenal stimulation does or does not affect hyperglycemia produced by various physiological means.

SUMMARY

The introduction of dilute HCl into the duodenum of unanesthetized dogs, nembutalized dogs, or nembutalized dogs with acutely inactivated adrenal glands does not lower the fasting blood-sugar level.

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THE DEPRESSANT ACTION OF STRYCHNINE ON THE SUPERIOR CERVICAL SYMPATHETIC GANGLION AND ON SKELETAL MUSCLE

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The well-known excitatory effect of strychnine in the central nervous system is in contradiction to the observations reported by Langley and Dickinson (1890), Langley (1918), Govaerts (1936) and Feldberg and Vartiainen (1934) that this drug has a depressant effect on sympathetic ganglia. In skeletal muscle Richet (1880), on injecting large doses of strychnine (5 cgm. per kgm. in rabbits), observed a paralyzing effect. Vulpian (1882) reported similar results. Lapicque (1907) and Bremer and Rylant (1924), working with excised neuromuscular preparations of the frog, attributed this paralyzing action of the drug to a failure of transmission of the nerve impulses. These results were later confirmed by other investigators.

It seemed of interest to study further the action of strychnine upon the transmission of nerve impulses in the superior cervical ganglion and in neuromuscular junctions, and to attempt to correlate the results obtained with the theory of chemical transmission of these nerve impulses by acetylcholine. The action of acetylcholine and prostigmin in relation to the effects of strychnine was therefore specially studied.

METHOD. Cats were used, under dial anesthesia (Ciba, 0.75 cc. per kgm. intraperitoneally). In some observations the animal was decerebrated or an Elliott preparation was made during brief etherization. If necessary a cannula was inserted into the trachea for artificial respiration.

When the superior cervical ganglion was studied, the isotonic contractions of the nictitating membrane were recorded by means of a lever having a 10-fold magnification and exerting a tension of 5 grams. In experiments with local application of the drugs the ganglion was exposed carefully to avoid any disturbance in its circulation.

To study the neuromuscular junctions the gastrocnemius-plantaris-soleus muscle was used, with the leg fixed by means of drills in the tibia. In some experiments the muscle was denervated by aseptic section

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of the sciatic 5 to 8 days before. The contractions of the muscles were recorded on a kymograph by attaching the tendon to the short arm of a writing lever pulling against a rubber band. The magnification was 6- to 10-fold.

For indirect stimulation the electrodes were shielded silver wires. When the muscles were stimulated directly, steel needles were inserted into their bodies and tendons.

Usually the stimuli employed were induction shocks from a Harvard coil, with 5 volts in the primary circuit. Repetitive stimulation at different frequencies was obtained from a multivibrator circuit. Condenser discharges of various capacities were also employed.

The substances injected were the following: strychnine (sulphate, Merck); acetylcholine (chloride, Merck); prostigmin (Roche); atropine (sulphate, Merck).

Results. A. *Electrical stimulation of preganglionic fibers.* Strychnine applied locally to the superior cervical ganglion or injected intravenously depresses the transmission of nerve impulses through the ganglion. The preganglionic fibers were stimulated maximally, on one side as a rule, for 2 to 5 seconds every 2 to 4 minutes. The nictitating membrane served as an indicator of the ganglionic response. In some animals the postganglionic fibers of the other side were similarly stimulated as a control.

When the ganglion was painted with a 1 or 2 per cent solution of strychnine, diminished contractions of the nictitating membrane were observed invariably. In some cases the responses after strychnine were only 20 per cent of the original magnitude. This effect was transient; after about 10 minutes the normal contractions were restored.

When the drug was injected intravenously, in doses of 0.005 to 0.04 gram for a 3-kilogram cat, the results were similar (fig. 1). The depression lasted longer for larger than for smaller doses. That this action was due to an impairment of transmission at the ganglion was shown by the lack of change of the responses of the nictitating membrane stimulated through postganglionic fibers.

Prostigmin was injected intravenously in order to learn whether it could abolish the action of strychnine. The doses employed were from 0.5 to 1 mgm. The injections were made during the period in which the strychnine depression was most noticeable. A restoration of the contractions was registered (cf. fig. 1). The membrane of the control side (postganglionic stimulation) showed no variation. The favoring effect of prostigmin lasted for about 10 minutes.

In some experiments submaximal stimulation of the preganglionic fibers was used in an attempt to demonstrate a possible excitatory effect of strychnine. An increase of the responses of the nictitating membrane, however, was never observed despite the different doses of the drug used.

B. *Stimulation of the ganglion by acetylcholine.* The technique described by Rosenblueth and Cannon (1939) for the stimulation of the superior cervical ganglion by intracarotid injection was used. The dose of acetylcholine employed was 40 γ in 0.2 cc. of distilled water. The responses of the membrane were double (Rosenblueth and Cannon, 1939), i.e., an initial sharp contraction, due to the effect on the ganglion, followed by a slower contraction due to direct action of the drug on the membrane. Painting the ganglion with strychnine, in the manner and with the doses described above, elicited a clear decline of the first component of the contraction, while the delayed component showed no change (fig. 2). The diminution

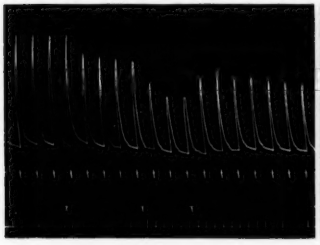

Fig. 1

Fig. 2

Fig. 1. Depressant effect of strychnine on the superior cervical ganglion stimulated through its preganglionic fibers. Isotonic contractions of the nictitating membrane. Upper signal: submaximal tetanic stimulation for 2 sec. Middle signal: intravenous injections; first and second, strychnine (0.01 gram); third, prostigmin (0.001 gram). Lower signal: 30-sec. time intervals.

Fig. 2. Depressant effect of strychnine on the superior cervical ganglion stimulated by intracarotid injections of acetylcholine. Isotonic contractions of the nictitating membrane. Upper signal: ganglion painted with strychnine (2 per cent solution). Middle signal: injections of acetylcholine (40 γ in 0.2 cc. saline). Lower signal: 30-sec. time intervals.

of the ganglion response lasted for about 5 minutes, whereupon the contractions evoked by acetylcholine recovered their initial magnitude.

C. *Indirect stimulation of skeletal muscle.* With single shocks applied to the sciatic nerve (one every half-minute), the intravenous injection of strychnine, in doses up to 0.04 gram, did not modify the tension developed by the gastrocnemius muscle.

When tetanic stimulation was used, two types of experiments were performed. In some cases strychnine (0.04 gram) was injected intra-arterially during a prolonged tetanus; the effects were similar to those caused by curare—i.e., a rapid drop of tension. In other instances tetanic stimulation was applied for 2 seconds every minute; an intravenous injection of strychnine produced a marked decline of the muscle responses (fig. 3).

The similarity between effects on sympathetic ganglia and on neuromuscular junctions led us to study the influence of prostigmin on the depressant action of strychnine. With doses of prostigmin equal to those used for the ganglion we were unable to find in muscle an antistrychnine effect. Indeed, in some experiments the depression was even greater after than before prostigmin. On the other hand, when small doses of prostigmin were injected (0.00025 to 0.0005 gram) a clear increase of the muscular responses was recorded, followed by a depression (fig. 3).

Fig. 3. Depressant effect of strychnine on the gastrocnemius stimulated indirectly. Upper signal: maximal tetanic stimulation of the sciatic nerve for 10 sec. Middle signal: intravenous injections; first, strychnine (0.04 gram); second, prostigmin (0.0005 gram). Lower signal: 1-minute time intervals.

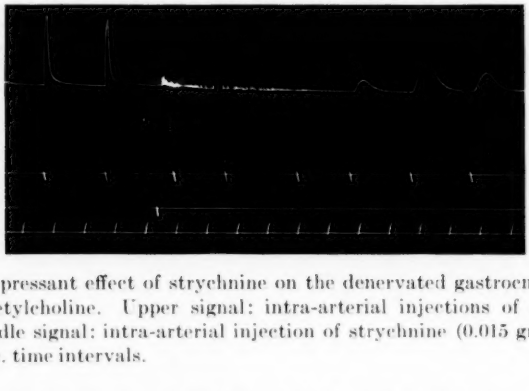

Fig. 4. Depressant effect of strychnine on the denervated gastrocnemius stimulated by acetylcholine. Upper signal: intra-arterial injections of acetylcholine (20 γ). Middle signal: intra-arterial injection of strychnine (0.015 gram). Lower signal: 30-sec. time intervals.

D. *Denervated muscles stimulated directly.* In order to exclude the possibility of direct action of strychnine on the contractile mechanism of skeletal muscles, observations were made on muscles whose motor nerves had been cut from 5 to 8 days before. The electrical stimuli were delivered through two needles, one in the belly and the other in the tendon of the denervated gastrocnemius-plantaris-soleus.

Injections of 0.24 gram of strychnine had no influence on the con-

tractions. It may be concluded that strychnine acts at the neuromuscular junction.

E. *Denervated muscles stimulated by acetylcholine.* The muscle used was the gastrocnemius-plantaris-soleus, denervated from 5 to 8 days before. The acetylcholine was injected into the femoral artery in doses from 20 to 40γ in 0.2 cc. Some of the experiments were performed after administration of atropine (1 mgm. per kgm.), in order to diminish the muscarine-like effects of acetylcholine.

When strychnine was injected intra-arterially (from 0.01 to 0.02 gram) the contractions caused by acetylcholine were diminished or abolished for about 5 minutes. After this period the responses to acetylcholine reappeared, but with changes in amplitude and shape (fig. 4). The contractions were smaller and more prolonged. When strychnine was injected intravenously the results were similar.

Discussion. The present experiments support the conclusion that strychnine exerts only a depressant action on the synapses studied. Under none of the experimental conditions and with none of the doses of the drug used was there an augmentor effect. The control observations localize the action at the junctions by eliminating direct influences on either the nictitating membrane or striped muscle.

This depressant action of strychnine is similar to that produced by curare at the same junctions. Other facts support the similarity. Rosenblueth and Luco (1937) showed that curare raises the threshold to acetylcholine of denervated muscles. Strychnine likewise raises the threshold to acetylcholine of both the superior cervical ganglion (fig. 2) and the denervated gastrocnemius-soleus (fig. 4). Bolly and Bacq (1938) observed a similar rise of the threshold of normal skeletal muscles. Prostigmin (Rosenblueth and Morison, 1937), like eserine (Pal, 1900), exerts a decurarizing action at neuromuscular junctions. The antagonism between prostigmin and strychnine (p. 280, fig. 3) resembles that between prostigmin and curare.

The similarity of effect of strychnine on the superior cervical ganglion and on the neuromuscular junctions supports Elliott's (1907) suggestion that the two synapses are analogous.

The depressant action of strychnine on the transmission of nerve impulses at the junctions studied (figs. 1 and 3) can be readily accounted for by the chemical theory of transmission. The threshold for injected acetylcholine is raised by strychnine; the threshold for acetylcholine liberated by nerve impulses is therefore also higher. The quanta of mediator released are consequently inadequate for transmission. Nachmansohn (1938) has pointed out that strychnine inhibits significantly the action of cholinesterase. Consequently acetylcholine would be less readily destroyed. It appears, however, that this protection of the mediator, which would lead to improved transmission, is overcome by the raised threshold

discussed above. On the other hand, a slowed destruction of acetylcholine would explain the prolonged responses of denervated muscles after strychnine (fig. 4).

The sharp contrast between the depressant effects of strychnine studied here and the excitatory action in the spinal cord, suggests that there may be fundamental differences in the mode of transmission of nerve impulses at different synapses.

SUMMARY

The effect of strychnine on the superior cervical ganglion and on the neuromuscular junction was studied in cats. In both synapses, whatever the doses used, only a depressant effect was observed. This depression occurred in the responses to nerve impulses (figs. 1 and 3) and to injected acetylcholine (figs. 2 and 4). Prostigmin counteracts partially the depressing effect of strychnine in either the ganglion or in striated muscle (figs. 1 and 3).

An explanation of these data on the theory of chemical transmission is attempted.

We wish to express our appreciation to Dr. A. Rosenblueth for his helpful suggestions throughout this work.

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QUANTITATIVE RELATION BETWEEN REACTIVE HYPEREMIA AND THE MYOCARDIAL ISCHEMIA WHICH IT FOLLOWS[1]

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Hilton and Eichholtz (1) reported a great increase in coronary flow when the heart in a heart-lung preparation was made anoxemic. We have recently observed a similar transient augmentation of coronary flow following temporary occlusion of the individual coronary arteries in an isolated heart preparation (2). These reactions are similar to the reactive hyperemia observed in the limbs of both animals and man. The quantitative correlation found by Freeman (3) between the reactive hyperemia and the period of ischemia in sympathectomized human hands, led us to look for a similar correlation in the denervated isolated dog heart. In order to study the uncomplicated responses of the musculature of the coronary vessels we have employed an isolated heart with ventricles fibrillating which we have shown (4) is suitable for such observation.

METHOD. In general the procedure previously used (4) was followed. The coronaries were perfused with defibrinated dogs' blood, keeping the inflow at a constant temperature (35–37°C.) and pressure (100–170 mm. Hg) throughout the experiment. Total coronary outflow was measured in a graduated cylinder as it came from the pulmonary artery. A cannula in the left ventricular cavity was used to drain away the Thebesian flow of the left heart and any possible leakage past the aortic valves. This left ventricular drainage was negligible in amount. In this isolated heart preparation with ventricles fibrillating, any change in coronary flow represents an active change in the calibre of the coronary vessels.

Seven animals were used in this study. In two, the isolated forelimb was simultaneously perfused under controlled conditions by the same perfusion system. Ischemia was produced for periods varying from 15 seconds to 5 minutes by suddenly clamping and later, suddenly opening the inflow tubing to the coronary vessels (and forelimb).

Once the data were obtained, the flow curve was plotted for each experiment and the periods of ischemia and reactive hyperemia were compared.

[1] Aided by The A. D. Nast Fund for Cardiac Research.

RESULTS. A typical experiment is shown in figure 2. Analysis of the data in these experiments showed:

1. There was little or no correlation between the total flow which would have occurred during the ischemic period (A, fig. 1) and any aspect of the

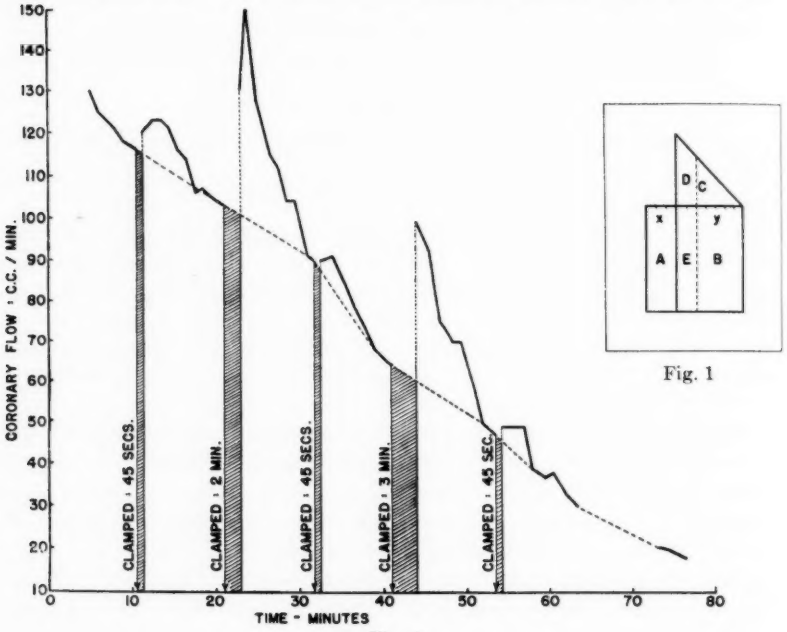

Fig. 1

Fig. 2

Fig. 1. Diagrammatic sketch of components of the ischemic and hyperemic periods, from which correlations between the two intervals were determined. A = volume of blood prevented from flowing during the ischemia. $B + E$ = control-level volume of blood flow during reactive hyperemic period. $C + D$ = total volume of blood in excess of control flow level following ischemia. D = volume of blood in excess of control flow level for first two-minute period following ischemia. E = control-level volume of blood flow for first two minutes following ischemic period. x = duration of ischemia. y = duration of reactive hyperemia. Discussed in text.

Fig. 2. Typical experiment showing effect on coronary outflow of periods of ischemia of 15,120, 15, 180 and 45 seconds, respectively. Comparison of the reactive hyperemia following the three 45-second intervals of clamping shows the typical progressive decrease in responsiveness encountered in this preparation. The lag in reaching maximum flow is best seen after the 120-second ischemia. Discussed in text.

period of reactive hyperemia. The total excess flow ($C + D$, fig. 1) was always less than that obstructed during the ischemia; it averaged 60 per cent of the latter.

2. A much better correlation existed between the duration of ischemia (x, fig. 1) and either the entire period of reactive hyperemia or some component of it. Thus:

a. Increasing the duration of ischemia increased the duration of the reactive hyperemia (y, fig. 1), but only slightly.

b. Increasing the duration of ischemia tended to increase the amount of excess flow during the period of reactive hyperemia but the correlation was rough.

c. The excess flow during the first two minutes of the reactive hyperemia (D, fig. 1) and the percentage increase in flow during this period $\left(\frac{D}{E} \times 100,\right.$

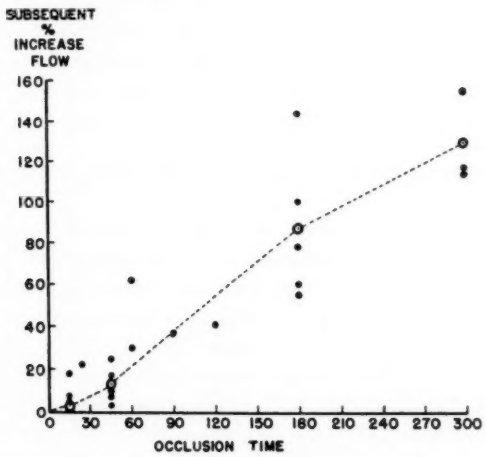

Fig. 3. Correlation of duration of ischemic period (abscissa) with the percentage increase in flow (ordinate) occurring during first two-minute period following ischemia. Dotted line is drawn through the mean values (shown by open circles) of all experiments.

fig. 1) showed even better quantitative correlation to the duration of the preceding ischemic period than the definite relationship shown by similar measurements for the entire period of reactive hyperemia. The percentage increase during the first 2-minute period gave the clearest correlation of all. In figure 3 are plotted the data of all observations on this correlation. It will be seen that the line connecting the means (shown by open circles) is practically straight.

3. The hyperemic response of the coronary vessels following ischemia was found to be much greater than that of the forelimb vessels in the two experiments where the simultaneous effects were compared.

4. The maximum flow in the reactive hyperemia did not always occur at once but tended to appear during the first minute after the ischemia was alleviated.

DISCUSSION. The tendency to lag in the dilator response of the coronary vessels is due to the method we employed in measuring coronary flow. The outflow persisted temporarily after the coronary inflow tube was clamped, and did not start at once on releasing this clamp, because of the emptying out of the blood in the coronary system in the former case and its refilling in the latter. Even after the coronary outflow has begun, this refilling process will cause the maximum outflow to be delayed. In view of this lag we used a 2 minute period rather than a shorter one in estimating the immediate response of the coronary vessels to ischemia.

The coronary blood flow was in excess of the amount necessary for maintaining constant the state of nourishment of the isolated fibrillating heart. Thus the blood leaving the coronaries still contained a large amount of oxygen. This normal luxus supply explains why the excess flow of blood during reactive hyperemia was always less than the flow which would have occurred during the preceding ischemia and partly explains the variable relation between the two. This normal variable luxus blood flow also accounts for the duration of ischemia being better correlated with the reactive hyperemia than the total flow interrupted during the ischemic period.

Furthermore, the poorer correlation of the total reactive hyperemia with the duration of ischemia as compared with the immediate response of the coronary vessels measured in the first two minutes, indicates that the stimulus for coronary dilatation is a diffusible substance which is not only destroyed locally in the presence of oxygen but is washed away during the reactive hyperemia. The amount washed away varies in different experiments because the rate of coronary flow shows progressive change even in the same preparation. The very poor correlation between duration of reactive hyperemia and that of the ischemic period supports this view.

The fact that a better correlation is obtained with the percentage increase in coronary flow than with the actual excess flow during the first two minutes of reactive hyperemia indicates that the dilatation is dependent in part on the responsiveness of the coronary vessels of the preparation. In the course of the life of the preparation, there is a tendency for the responsiveness of the coronary vessels to diminish as shown by the decreased dilatation obtained with equivalent amounts of epinephrine as an experiment progresses. Variations in the responsiveness of the coronaries occur also from animal to animal.

If the reactive hyperemia were just equal to that needed to make up the deficit acquired in the ischemic period, a close correlation should have been found between the total excess flow in reactive hyperemia and the duration of ischemia. The lack of such a correlation suggests that the former is in excess of that needed to make up the deficit acquired in the ischemic period.

Our results, therefore, suggest that:

1. There is normally a luxus coronary blood supply of variable extent in this preparation.

2. The reactive hyperemic response is in excess of that needed to make up the deficit sustained during the preceding ischemia.

3. The stimulus for reactive hyperemia appears to be a dilator substance eliminated by oxygen and sufficiently diffusible to be washed away in the blood stream.

4. The reaction of the coronary vessels depends not only on the amount of this substance liberated during ischemia but also upon the reactivity of the coronary vascular system which is variable in the course of an experiment and in different experiments.

These facts doubtlessly apply also to the coronary vessels under more normal conditions and perhaps to other vascular beds. Further, the results of our experiments indicate that limb vessels are less responsive to ischemia than the coronary vessels. This is important for compensating the effects of ischemia, since as Katz and Long (5) have shown, the beating heart is more susceptible to the ill effects of ischemia and anoxemia than is skeletal muscle. This lesser responsiveness to ischemia of the limb vessels than of the coronary vessels may be due to either a smaller amount of dilator substance being formed or to a lesser responsiveness of the vessels to equal amounts of dilator substance.

In the heart, therefore, there exists a local mechanism independent of nerves, or extracardiac humoral mechanisms by which rapid and large adjustments of coronary flow can be made in accordance with the need for blood (cf. also Rein, 6). This mechanism as in the case of other organs (Rein, 7; Rein and Schneider, 8; Hess, 9; Fleisch, 10) appears to depend on the accumulation of relatively easily diffusible metabolites readily eliminated by oxygen. Whatever the dilator substance may be, the mechanism augmenting the coronary supply is the inability of the existing coronary flow to meet myocardial needs. This mechanism would come into play regardless of whether the deficiency of blood resulted from a decrease in the quantity of coronary flow by an external factor, from a defect in the quality of the blood or from an increase in the energy expended by the myocardium. The effectiveness of this dilator mechanism would of course depend on the responsiveness of the coronary vessels to it and to the magnitude and duration of the coronary flow insufficiency. Obviously should the coronary vessels be unresponsive, or the ischemia be the result of a persistent complete obstruction of one or more coronary vessels, this compensatory reaction would fail and either heart failure or local infarction of the heart muscle would develop. With less severe or less permanent insufficiency and in responsive vessels, the reactive hyperemia is important in accelerating the restoration of the myocardium to its normal state.

SUMMARY

The quantitative effect on coronary flow of different periods of myocardial ischemia was observed in an isolated preparation of the dog heart with ventricles fibrillating. Blood-flow through the fore-limb of the same animals was studied simultaneously under the same conditions. In this preparation coronary flow is determined entirely by active changes in the coronary vessels.

In the heart, correlation of the components of the ischemic periods with those of the subsequent hyperemic periods and analysis of the relationships found, led to the following conclusions:

1. In this preparation, the coronary blood supply is greater than that necessary to meet myocardial needs.

2. The hyperemia due to the ischemia is more than adequate to make up for the myocardial deficit acquired.

3. The cause of the hyperemia seems to be an easily diffusible dilator substance which is eliminated in the presence of oxygen.

4. The degree of hyperemia not only varies with the duration of the ischemia and hence with the accumulation of the dilator substance, but also with the responsiveness of the coronary vessels to this substance.

The coronary vessels are decidedly more reactive to ischemia than are the limb vessels.

The importance of reactive hyperemia as a mechanism operating to compensate for any inadequacies in coronary flow in the intact animal is emphasized, and the bearing of these data on reactive hyperemia in other vascular beds is suggested.

We are indebted to other members of the department for technical help with the experiments.

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INFLUENCE OF THE COMPOSITION OF THE DIET ON THE THIAMIN REQUIREMENT OF DOGS[1]

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Many investigators (Evans and Lepkovsky, 1928, 1929; Salmon and Goodman, 1937; Stirn, Arnold and Elvehjem, 1939) have demonstrated that rats fed diets high in fats show a decreased requirement for thiamin (vitamin B_1). This sparing action of fat has not been demonstrated for other species. Westenbrink (1936) was unable to show a reduced vitamin requirement in pigeons fed high fat diets. In continuation of our studies on the thiamin requirement of various species (Arnold and Elvehjem, 1938a; 1938b) we were interested in determining whether the thiamin requirement of dogs could be expressed in a manner similar to that previously reported for rats and chicks, namely, approximately 80 micrograms per 100 grams of diet and whether an increased fat intake would decrease the level of vitamin necessary.

EXPERIMENTAL. The composition of the diets used in these studies is given in table 1. The purified casein was prepared from domestic crude casein by solution in dilute ammonium hydroxide, precipitation with dilute hydrochloric acid and washing with water. The process was repeated twice. The yeast[3] was autoclaved for five hours at 15 pounds pressure. Salts 3 is the salt mixture described by Phillips and Hart (1935) with added manganese (2.55 parts $MnSO_4 \cdot 4\ H_2O$ added to 2232.4 parts salts 1). The steamed bone meal[4] was the ordinary commercial grade. Each diet contained one part of liver extract[5] which had been treated with sodium sulfite to destroy the thiamin and which served as an added source of the B vitamins other than thiamin. The sulfiting procedure will be described in a separate communication.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] Wilson and Company Fellow.

[3] Strain C, Anheuser-Busch, St. Louis, obtained through the aid of Dr. P. L. Pavcek.

[4] Kindly supplied by Dr. E. H. Harvey, Wilson and Company, Chicago.

[5] The liver extract was the material which remained in solution after the precipitation of the P. A. factor by 92 per cent alcohol. Supplied by Dr. David Klein, Wilson Laboratories, Chicago.

The mongrel pups used in this study were all males. Dogs 1 and 2, as well as dogs 3, 4 and 5, were littermates. The dogs were placed on experiment at six weeks of age. The dry diets were fed *ad libitum* to the dogs. Water was supplied in porcelain jars twice daily and was available at all times.

In the early stages of the experiments with dogs 1 and 2 polished rice was used instead of sucrose in ration 280. The dogs continued to grow on the ration which indicated that the polished rice supplied more thiamin than was permissible in these studies. This source of carbohydrate was, therefore, replaced by sucrose. With this change in the ration the dogs on the basal were rapidly depleted of their thiamin reserves as indicated in the records which follow below.

RESULTS. *Dog 1* (fig. 1). When the dog had declined somewhat in weight and exhibited a decreased food intake due to the thiamin deficiency,

TABLE 1

Composition of the diets

RATION		CONSTITUENTS*									
		Sucrose	Autoclaved lard	Purified casein	Autoclaved yeast	Salts 3	Steamed bone meal	Cottonseed oil	Cod liver oil	"fat"	"non-fat"
280		72		18	4	2	2	1	1	2	94
281	Parts	36	16	18	4	2	2	1	1	18	58
	Per cent	45	20	22.5	5	2.5	2.5	1.25	1.25	22.5	72.5
282	Parts		32	18	4	2	2	1	1	34	22
	Per cent		53.3	30	6.7	3.3	3.3	1.7	1.7	56.5	36.7

* Each diet contained, in addition, one part of sulfited liver extract.

the basal was supplemented with 100 micrograms per cent thiamin chloride.[6] There was an immediate growth response and an increase in the food intake as seen in the figure. This level of thiamin chloride satisfied the requirement of the dog during a period of four months.

At 115 days the vitamin supplement was decreased to 75 micrograms per cent. This level of vitamin maintained the dog in good condition for 30 days. There was some variation in food intake, but this was probably related to overfeeding.

At 145 days the thiamin content of the ration was decreased to 50 micrograms per cent. This level of the vitamin was unable to maintain the dog as evidenced by the steady decrease in food intake of the dog after five days on the lowered vitamin level. The dog did not lose weight

[6] This study was materially facilitated by a generous supply of synthetic thiamin chloride by Merck and Company, Rahway, N. J.

to any marked degree although the appearance of the animal indicated general debility. Since it was desired to demonstrate a weight loss, the vitamin supplement was not increased in time to save the dog.

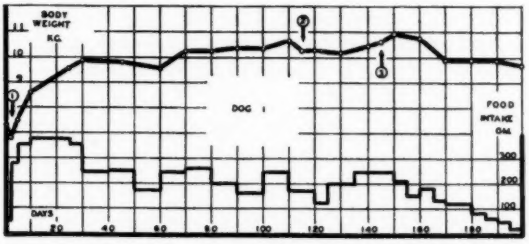

Fig. 1. Growth record and food consumption of dog 1 fed ration 280 to deplete the thiamin reserves and then supplemented with thiamin.

1. Diet supplemented with 100 micrograms per cent thiamin chloride.

2. Thiamin content of the diet decreased to 75 micrograms per cent.

3. Vitamin supplement decreased to 50 micrograms per cent.

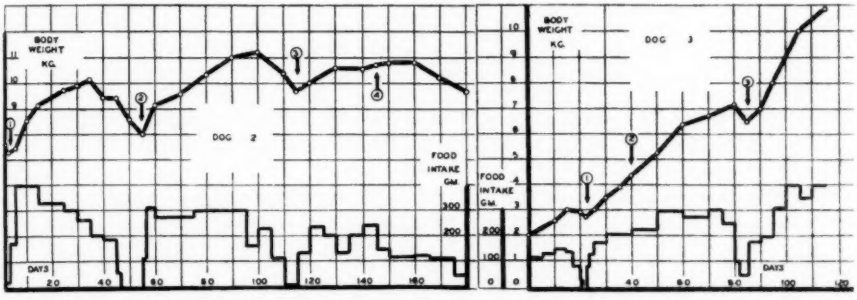

Fig. 2 Fig. 3

Fig. 2. Record of dog 2 fed ration 280 to deplete the thiamin reserves and then fed the basal supplemented with the antineuritic vitamin.

1. Diet supplemented with 50 micrograms per cent thiamin chloride.

2. One milligram of thiamin chloride injected subcutaneously; vitamin supplement continued as before.

3. Thiamin chloride supplement increased to 75 micrograms per cent.

4. Vitamin content of the diet decreased to 50 micrograms per cent.

Fig. 3. Record of dog 3 fed the unsupplemented basal ration for 22 days then given thiamin supplements as indicated.

1. Diet supplemented with 75 micrograms per cent thiamin chloride.

2. Vitamin supplement decreased to 50 micrograms per cent.

3. Thiamin content of the diet increased to 75 micrograms per cent.

Dog 2 (fig. 2). The major portion of the thiamin reserves of this dog was depleted by restricting the dog to ration 280, as in the case of dog 1, until a weight loss and decreased food intake were observed. The diet

was then supplemented with 50 micrograms per cent thiamin chloride. The amount of food voluntarily consumed by the dog increased and was maintained at a normal level for a period of two weeks. The weight of the dog rapidly increased during this time. The food intake then decreased until the 47th day when the dog stopped eating completely. The amount of food consumed by the dog supported growth through the 35th day but the weight of the dog decreased sharply thereafter.

At 54 days 1 mgm. of thiamin chloride was injected subcutaneously. The effect of this injection coupled with the fact that the ration contained 50 micrograms per cent thiamin chloride protected the dog from the anorexia associated with the vitamin deficiency for approximately 40 days. After this time there was another drop in food intake and a decrease in body weight.

At 115 days the vitamin supplement was increased to 75 micrograms per cent. The favorable response to the increased level of thiamin was evident from the marked increase in food intake and a slight rise in body weight.

A decrease in the thiamin content of the diet to 50 micrograms per cent at 145 days led to a decrease in the food intake and a loss in body weight which terminated in the death of the dog.

Dog 3 (fig. 3). The dog was fed ration 280 for 22 days to deplete the thiamin reserves. The food consumption at this time had decreased markedly. The dog responded favorably to a thiamin chloride supplement added at 75 micrograms per cent.

The vitamin content of the ration was decreased to 50 micrograms per cent at 40 days. The dog grew well during the following 3-week period but more slowly thereafter. The food consumption was uniformly good during this period of growth. The level of thiamin in the diet was insufficient to maintain the dog, however, and the food intake of the dog dropped sharply at the 80th day. There was a concomitant loss in weight.

At 85 days the vitamin content was increased to 75 micrograms per cent. The food consumption of the dog voluntarily increased which was followed by an excellent increase in the weight of the dog.

Dog 4 (fig. 4). This dog received ration 281, which contains 22.5 per cent fat, supplemented with 54.3 micrograms per cent thiamin chloride at the start of the experiment. The vitamin supplement may be expressed in terms of the total ration as above or in terms of the non-fat constituents of the diet as given in table 1. On the basis of the non-fat constituents the dog received 75 micrograms per cent thiamin. The growth of the animal was good on this ration.

The vitamin supplement to the diet was decreased to 36.2 micrograms per cent (50 micrograms per cent in terms of the non-fat constituents) at

40 days. There was a decreased food intake after 10 days which was followed by a loss in weight of the dog.

To further demonstrate that the loss in weight was a thiamin effect, the vitamin supplement was increased at 65 days. The dog responded favorably to the increase in the thiamin supplement to 54.3 micrograms per cent or 75 micrograms per cent on the basis of the non-fat constituents.

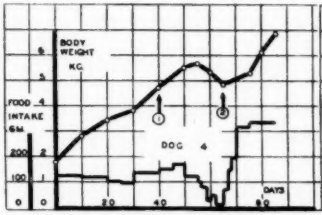

Fig. 4

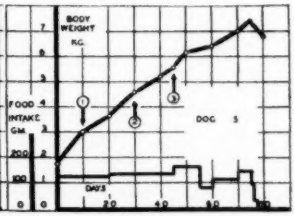

Fig. 5

Fig. 4. Record of dog 4 started on ration 281 supplemented with 54.3 micrograms per cent of thiamin chloride or 75 micrograms per cent thiamin chloride on the basis of the non-fat constituents.

1. Thiamin chloride supplement decreased to 36.2 micrograms per cent or 50 micrograms per cent on the basis of the non-fat constituents.

2. Thiamin chloride supplement increased to 54.3 micrograms per cent thiamin chloride or 75 micrograms per cent on the basis of the non-fat constituents.

Fig. 5. Record of dog 5 started on ration 281 supplemented with 54.3 micrograms per cent thiamin chloride or 75 micrograms per 100 grams of non-fat constituents of the diet.

1. Ration modified by isocalorically substituting half of the sucrose in ration 281 with fat and the thiamin chloride supplement added at 43 micrograms per cent or 75 micrograms per 100 grams of non-fat constituents of the ration.

2. Ration further modified by withdrawal of the remainder of the sucrose component, isocalorically replaced by autoclaved lard (ration 282), and the thiamin chloride supplement supplied at a 27.5 micrograms per cent level or 75 micrograms per 100 grams of non-fat constituents of the ration.

3. Vitamin supplement decreased to 18.3 micrograms per cent or 50 micrograms per 100 grams of the non-fat constituents of ration 282.

Dog 5 (fig. 5). This dog was started on ration 281 supplemented with 54.3 micrograms per cent thiamin chloride, 75 micrograms per cent on the basis of the non-fat constituents. The dog grew well on this diet.

The ration was modified at 10 days so that three-fourths of the sucrose had been isocalorically replaced by fat (24 parts of fat for 54 parts of sucrose). The ration was supplemented with 43 micrograms per cent thiamin chloride, 75 micrograms per cent in terms of the non-fat constituents. The dog continued to eat and grow at a rate which compared favorably with that of dog 30.

At 30 days the diet was modified for the second time so that the dog

was then fed ration 282, which contains 56.5 per cent fat, supplemented with 27.5 micrograms per cent thiamin chloride or 75 micrograms per cent in terms of the non-fat constituents. The food consumption and growth of the dog were not affected by the change in the ration.

At 45 days the vitamin supplement was decreased to 18.3 micrograms per cent or 50 micrograms per cent in terms of the non-fat constituents of ration 282. The record which followed was similar to that obtained with the other dogs. The food consumption remained at a high level for 10 days. There was a temporary drop during the next 5 days, a rise in the food intake for a 2-week period and then a rapid decrease. The amount of food consumed by the dog supported growth for 4 weeks. There was a sharp drop in body weight alone with the decrease in food intake after the 75th day.

Discussion. The results with dogs 1, 2 and 3 clearly demonstrate that at least 75 micrograms per cent thiamin chloride in the diet is necessary to meet the normal requirement for this vitamin. A reduction in the thiamin chloride supplement to 50 micrograms per cent resulted in the appearance of the anorexia associated with a thiamin deficiency. If the thiamin requirement for the different dogs is expressed in terms of body weight we find that there are great variations for the different dogs at the various stages in the experimental period. The thiamin intake per kilogram of body weight for dog 3 fed 75 micrograms thiamin chloride per 100 grams of ration 280 between the 25th and 40th days on experiment ranged between 38 and 42 micrograms or 13–14 I.U. per day. For the period between the 40th and 75th days the vitamin intake was between 21 and 26 micrograms or 7–9 I.U. per kilogram per day. The vitamin intake of dog 1 fed ration 280 supplemented with 75 micrograms per cent thiamin chloride was 13 to 18 micrograms or 4–6 I.U. of thiamin chloride per kilogram per day between the 115th and 145th days. There was a slight drop during the 5-day period, 120–125 days, during which the dog was off feed somewhat and ingested 9 micrograms of thiamin chloride per kilogram per day. The thiamin intake for dog 1 when fed ration 280 supplemented with 75 micrograms per cent thiamin chloride was thus lower per unit body weight than the vitamin intake of dog 3 fed ration 280 supplemented with 50 micrograms per cent thiamin chloride during a 6-week period. Since the food requirements for growth are higher than those for maintenance per unit body weight, the method used in this study wherein the vitamin was fed in the ration automatically took care of the increased thiamin requirement per unit body weight in the growing dog because the increased food intake supplied more vitamin. The results, therefore, warrant the conclusion that the thiamin requirement is best stated as percentage of the diet. The results can hardly be considered to support

the observations of Dann and Cowgill (1934) that the thiamin requirements during growth are 3 to 5 times the requirements for maintenance.

This study together with the previous ones demonstrates that the thiamin requirements of the rat, chick and dog are markedly similar when they are stated as per cent of the diet. These results are in agreement with the conclusions independently reached by Williams and Spies (1938). These authors have stated that "all species of animals require somewhat less than one part per million of thiamin in the food when carbohydrate predominates in the diet to the degree which is usual for most species." The oft-repeated observation of Osborne (Cowgill, 1932; 1938) to the effect that "man cannot possibly require relatively as much vitamin B as rat experiments would suggest" is therefore misleading since the requirement is similar when expressed in terms of the diet. This study indicates that the same level of thiamin in the ration will satisfy the thiamin requirement during growth and maintenance.

The results with dogs 4 and 5 are in accord with the concept that the thiamin requirement is greatly reduced when fat is metabolized and that the thiamin requirement may best be stated in terms of the non-fat constituents of the diet. This conclusion is at variance with the results submitted by Cowgill, Deuel and Smith (1925). In their studies on the thiamin requirements of various species approximately half the calories in the rations for the dogs were supplied by fat. In spite of this, the thiamin values obtained with the dogs were calculated to be in accord with the thiamin values determined with other species. This would indicate that a several-fold error contributed by any one species is not significant in Cowgill's formula. The results with fats further do not substantiate the statement (Cowgill, 1934) that "the 'vitamin-sparing action of fats' may after all prove to be due to the actual presence of vitamin B in the fat." Williams and Spies (1938) concluded that lard does not contain any thiamin. The lard used in these studies was nevertheless autoclaved as an added precaution against thiamin contamination. The dogs did not dislike the heat-treated article.

The polyneuritic symptoms of dogs as we have observed them vary slightly from those reported by Cowgill (1921). The dogs suffering from a severe thiamin deficiency do not have a foul breath while we have noted that nicotinic acid deficient dogs do show this condition. This observation is in accord with the conclusions of Williams and Spies (1938) that the deficiency studied by Cowgill was multiple in nature.

The results with dogs 4 and 5 clearly demonstrate that in the presence of increased amounts of fat in the diet, the thiamin requirement may be reduced to almost a third of the level required in the low fat diets. However some thiamin is necessary even in the high fat diets. This, therefore,

indicates that the diets used in the studies on the high fat rations with rats reported elsewhere (Stirn, Arnold and Elvehjem, 1939) supplied small amounts of the antineuritic factor. The amount of vitamin required to satisfy the requirements for rats on a diet rich in fat may be of the order of 1.0 to 1.5 micrograms per day.

The studies here indicate, as have those with the rats, that the isocaloric substitution of fats for carbohydrates in the diets is a nutritionally sound procedure. Assuming that the initial ration is balanced, the balance is necessarily retained even when large amounts of fat are substituted for the carbohydrate in the diet. The method is limited in the sense that the amount of fat which can be incorporated into the ration may not be as great as may be desired. Since the dogs fed the high fat diets ate and grew well when supplied with protective levels of thiamin in the diet, there is no basis for restricting the fat content of the rations of dogs to a low level.

The studies of Jolliffe and Goodhart (1938) may be interpreted somewhat differently in the light of these results. The diet used by these investigators for studies with human subjects contained 382 grams carbohydrate, 79 grams protein, 144 grams fat and 474 micrograms thiamin. The thiamin content was calculated from the values in Cowgill's tables (1934). The diet was calculated by these investigators to supply only 50 per cent of the predicted thiamin requirement of a subject weighing 70 kgm. The conclusions reached in this communication indicate that 474 micrograms of thiamin per 461 grams of non-fat diet should satisfy the thiamin requirement. Since the literature review of Williams and Spies (1938) indicates that the thiamin values submitted by Cowgill are high, it is likely that the thiamin content of the diet as calculated by Jolliffe and Goodhart was also high. Since no experimental data are submitted in the report of Joliffe and Goodhart it is not possible to assess the results of feeding their diet to human subjects.

If the conclusions reached in this study are extended to human nutrition and if it is accepted that 75 to 100 micrograms per cent thiamin in the diet is sufficient to meet the demands, then the thiamin required for a 70 kilogram man, whose food intake is 3000 calories per day (750 grams of fat-free diet), should be 560 to 750 micrograms (187 to 250 I.U.). Fats in the diets would correspondingly lower the requirement. Van Veen (1935) has estimated that 150 I.U. per day represents the minimum for protection against beriberi while 200 I.U. is regarded as a safe level for orientals. These results are in agreement with the studies on 193 normal infants (Colby, Macy, Poole, Hamil and Cooley, 1937) which indicated that the average weight and height of the infants which received 1.0 to 1.5 micrograms of thiamin per gram of solids was not significantly different from those which received 3.0 to 4.5 micrograms of thiamin per gram of

solids. Since the thiamin is related to food intake rather than to the physiological state, our studies are in agreement with the recommendations of the League of Nations Health Committee (1935) which evaluated the requirement during a caloric intake of 3000 to 3500 calories during pregnancy and lactation to be 150 to 250 I.U. The Committee on Nutritional Problems of the American Public Health Association (1934–5) recommended approximately 200 I.U. of thiamin as more than the minimum requirement for the adult man. The Council on Pharmacy and Chemistry of the American Medical Association (1936) has also concluded that 200 I.U. of thiamin daily will meet the adult requirement.

SUMMARY

Results are presented to show that growing and mature dogs can be protected from the anorexia associated with a thiamin deficiency by the inclusion of 75 micrograms of thiamin chloride per 100 grams of a fat-low basal ration.

When the fat content of the diet was increased to 56.5 per cent, by isocalorically replacing the sucrose of the diet with fat, the thiamin requirement of the dogs was decreased to approximately one-third (27.5 micrograms per cent) of the former level. This level of the vitamin was 75 micrograms per 100 grams of the non-fat constituents of the high fat ration. The dogs readily consumed and grew well on the high fat diets when the latter contained adequate amounts of the antineuritic factor.

Experimental results are offered to demonstrate that the requirement for thiamin by most species of animals may be simply stated as approximately one microgram of the vitamin per gram of dietary solids. In the presence of increased amounts of fat in the diet the requirement is correspondingly reduced.

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ADRENALINE HYPERGLYCEMIA: PROPORTIONALITY WITH DOSE

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There is a large literature dealing with the effect of adrenalin on blood sugar level which it is unnecessary to refer to in the present connection except to state that, so far as has been discoverable by reasonably diligent search, the relationship to be set forth here, viz., proportionality between dosage and hyperglycemia, appears hitherto to have escaped systematic exploration.

These results will show increase in hyperglycemic effect to a maximum, as rate of intravenous injection of adrenalin is increased within moderate range of dosage; beyond this, hyperglycemia not only does not further increase, but is actually reduced.

METHODS. Cats anesthetized with chloralose (0.1 gram per kilo, subcutaneously, or intramuscularly) were used exclusively.

As soon as anesthesia was complete the animals were fastened on an electrically heated holder, by which rectal temperature was kept normal with very little fluctuation. Further preparation consisted merely of insertion of the following cannulae: tracheal; one carotid for blood sampling and the other for registration of blood pressure; one in a superficial branch of the femoral vein for injection. Following this preparation, 15 to 20 minutes were allowed for stabilization before taking the first blood sample.

Blood sampling. Normal blood: in the beginning two normal blood samples, 15 to 20 minutes apart, were taken preceding injection in order to establish the magnitude of spontaneous change; these results soon showed such stability of the resting normal blood sugar level that thereafter, to conserve blood volume, one normal sample was considered sufficient.

Following injection, samples were taken, immediately, and 10 minutes, and 30 minutes after its completion.

Samples were obtained from a large cannula holding slightly over 2 cc. in one of the carotid arteries.

Blood analysis. Two cubic centimeters of blood were removed by pipette from the carotid cannula; blood sugar was determined by the method of Hagedorn and Jensen applied to a Folin-Wu tungstic acid filtrate.

Injection. Parke-Davis adrenalin chloride was diluted with neutral (pH 7.3) isotonic NaCl solution so the amount to be injected per minute

was contained in 1 cc. Injection into the femoral vein, was always for 5 minutes, 1 cc. per minute, by hand from a 5 cc. syringe, with care to maintain uniformity of rate. The rates employed, with the number of experiments (in parenthesis) were, milograms per kilo per minute: 0.00025 (10); 0.00050 (6); 0.00100 (9); 0.00200 (14); 0.00400 (13); 0.00700 (10); 0.01000 (6).

Control injections of isotonic NaCl (10 experiments) were similar in amount, rate and duration.

RESULTS. *Normal Blood Sugar Level. Intra-individual stability:* Reference has been made to the initial procedure of taking two normal blood samples, 15 to 20 minutes apart, preceding injection; this was done with the 27 animals subsequently receiving adrenalin, 0.002 and 0.004 mgm. per kilo per minute, with the average result that the blood sugar concentrations of the first and second samples for the first group were 131 and 132 mgm. per cent respectively; and for the second group, 129 mgm. per cent for both. This seemed to indicate that under the conditions of these experiments the resting blood sugar level was sufficiently stable to eliminate danger of spontaneous change interfering with interpretation of the results following injection; in the remaining experiments, therefore, one determination of the resting level was considered sufficient.

Inter-individual variability: In spite of this steadiness of the resting blood sugar level for individual animals, the inter-individual variability was large, although all animals were treated in as uniform manner as possible; thus, for the different groups, the range and average, with the total number of experiments in each are shown in columns (2), (3) and (4) of table 1.

A similar normal variability and almost identical average have been described before (Griffith, 1923) and are, therefore, apparently to be expected under conditions similar to these.

Within any one group there is no correlation between initial, resting blood sugar level and magnitude of increase produced by adrenalin; nevertheless, it seemed worth while to fortify the conclusions based on the unselected totals, with their large variability of average resting level, by evidence having more homogeneous foundation. For this purpose those experiments of each group tending to raise or lower the group average above or below the grand average of 146 mgm. per cent, were eliminated; this left the number of experiments in each group as shown in column (6) and the average, resting blood sugar values as in column (5).

Evidence based on these two sets of normal figures will be referred to in what follows as total averages and corrected averages, respectively.

The Effect of Control Injection of Isotonic NaCl. The averages of the 10 experiments in which isotonic NaCl was injected in amount similar to that serving as vehicle for adrenalin in the remaining experiments showed blood sugar increases at the end of the 5-minute injection, and 10, and 30

minutes following, of 3, 7 and 13 per cent. Whether this is a specific salt effect, or, as is probably more likely, the result of stimulation and hemorrhage involved in obtaining the blood samples, was not determined and for present purposes is immaterial.

These control values have been used as the initial points for the curves of figure 1.

The Effect of Adrenalin Injection. The total and the corrected averages show similar effects: In spite of the divergent normal values, reference to figure 1 will show that the actual increases in milligrams per cent based on the total averages are practically the same as the percentage increases based on averages corrected to 146± mgm. per cent. The one or two small differences between the two sets of curves are too insignificant to have any

TABLE 1

GROUP RECEIVING	UNSELECTED TOTALS			SELECTED FOR 146± AVERAGE	
	Normal blood sugar values		Number of experiments	Average	Number of experiments
	Range	Average			
(1)	(2)	(3)	(4)	(5)	(6)
mgm./kgm./min.	*mgm. per cent*	*mgm. per cent*		*mgm. per cent*	
0.00025	117–206	162	10	146	7
0.00050	98–195	139	6	147	5
0.00100	92–152	126	9	143	4
0.00200	75–265	132	14	146	11
0.00400	102–174	129	13	144	7
0.00700	100–215	167	10	145	6
0.01000	143–219	169	6	148	4
NaCl controls.....	93–228	144	10	144	10
Grand average....		146			

bearing on their general contour; which may safely be taken as specifically the result of adrenalin in varying concentration rather than of the initial blood sugar level at the time of injection. That is, and it would seem a minor point of some interest in the mechanism of carbohydrate mobilization by adrenalin, whether resting blood sugar level is high or low within limits of normal variation, further increase is proportional to rate of adrenalin injection irrespective of absolute blood sugar concentration.

The minimal effective rate of injection: Reference to figure 1 will show that injection at the rate of 0.00025 mgm. per kilo per minute has an effect only so slightly greater than that obtained with the saline controls as to point to the next larger rate, 0.00050 mgm. per kilo per minute, as the real threshold for blood sugar elevation. This is twenty times the thresh-

old reported for normal men (Cori and Buchwald, 1930) and from five to ten times that for unanesthetized rabbits (Cori, Cori, and Buchwald, 1930). If it may be assumed that the threshold for unanesthetized ani-

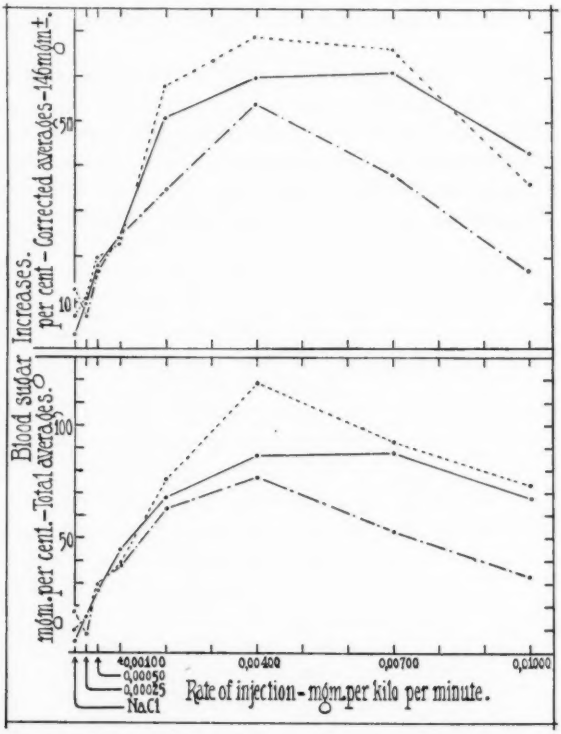

Fig. 1. Blood sugar increases produced by injection of adrenalin at rates between 0.00025 and 0.01000 mgm. per kilo per minute for 5 minutes. It will be noted that these curves are not the ordinary blood-sugar curves which they much resemble, but represent blood sugar increases plotted against dosage. Solid line: blood sugar values at the end of the 5-minute injection. Dotted line: blood sugar values 10 minutes after injection. Dashed line: blood sugar values 30 minutes after injection. Upper set of curves: percentage increases of blood sugar based on corrected normal averages of 146 ± mgm. per cent. Lower set of curves: actual increases in milligrams per cent based on the unselected, total averages. The initial point of each curve is the value resulting from control injection of isotonic NaCl in amount serving as vehicle for the adrenalin of the other injections.

mals is very probably not higher in the cat than in the rabbit, this would indicate that chloralose slightly depresses the mechanism of carbohydrate mobilization by adrenalin.

The maximum blood sugar increase is characteristically not co-terminous with adrenalin injection. Thus, in the figure, the dotted line representing blood sugar values 10 minutes after injection has stopped, is, at nearly all rates of injection, the highest of the three curves. Either adrenalin remains in the circulation or in the tissues and is not destroyed as rapidly as is commonly supposed; or events set in motion by it, in so far as they bear on blood sugar elevation, persist with a certain inertia into the immediate post-injection period.

Proportionality Between Blood Sugar Increase and Adrenalin Concentration. The most unexpected feature of these results is the evidence that beyond a certain optimum rate of injection, which from these data is in the neighborhood of 0.004 mgm. per kilo per minute, blood sugar elevation is not only increased no further but is actually diminished. It will be seen that nearly doubling this rate of injection, 0.007 mgm. per kilo per minute, has practically no greater effect on the elevation reached at the end of the 5-minute injection; whereas the blood sugar levels 10 and 30 minutes later are distinctly lowered. And finally, 0.01 mgm. per kilo per minute is definitely less effective at all three time intervals. Rate of intravenous injection greater than this would be of no physiological interest; this dosage was fatal for 2 animals out of 12; blood sugar determinations were made on only 6 of the other 10; although all 10 of these survived in good condition, there seemed no point to pushing the injection beyond this rate of apparently maximum toleration. No untoward effects of any kind, unless that described here is so interpreted, ever accompanied the use of 0.007 mgm. per kilo per minute.

Discussion. The increasing effectiveness of adrenalin in elevating blood sugar level as shown by these data for rates of injection between 0.00025 and 0.00400 mgm. per kilo per minute for 5 minutes is probably to be expected according to any theory of adrenalin action. It is unfortunate data are not available for the response to rates of injection between 0.004 and 0.007 mgm. per kilo per minute; but from the shape of the curves based on the present facts it would seem doubtful whether the rate of injection of maximum effectiveness would be found to be much above the lower of these.

The diminishing effectiveness of the higher rates of injection, 0.007 and 0.010 mgm. per kilo per minute, is something which seems until now to have escaped notice. And no explanation of this reversal with larger doses is implicit in any of the proposed theories of adrenalin action. Thus until a similar reversal is demonstrated for hepatic glycogenolysis, suppression of carbohydrate utilization, vasoconstriction and anoxemia, acapnia, hypercapnia and increased blood acidity, or any of the many factors that have been put forward from time to time to explain the hyperglycemia, there can be no profit in attempting to reconcile the present findings with

any of these proposed explanations. At the most it might be ventured that the hyperglycemia is a result of the operation of several factors such as glycogenolysis, metabolic rate and effective blood supply to the tissues, whose algebraic sum passes through a maximum under the conditions of these experiments when adrenalin is injected intravenously at the rate of approximately 0.004 mgm. per kilo per minute.

SUMMARY

Intravenous injection of adrenalin at rates of 0.00025, 0.00050, 0.00100, 0.00200, 0.00400, 0.00700 and 0.01000 mgm. per kilo per minute for five minutes affects blood sugar level of chloralose anesthetized cats as follows:

a. The approximate minimal effective dose is 0.00050 mgm. per kilo per minute.

b. Maximum blood sugar level is usually attained in the 10-minute interval following the 5-minute injection period.

c. Hyperglycemia increases with dose up to 0.004 mgm. per kilo per minute; the two rates of injection beyond this, 0.007 and 0.010 mgm. per kilo per minute, produce progressively decreasing augmentation of blood sugar level.

The difficulty of reconciling such a result with the operation, singly, of any one of the factors hitherto advanced to account for adrenalin hyperglycemia warrants the tentative suggestion that explanation should be sought in the interaction of several of the well-known physiological reponses to this hormone.

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THE EFFECT OF PIPERIDO-METHYL-3-BENZODIOXANE (933F) ON THE HEART OF THE FROG AND THE CAT

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In 1937, Shen reported experiments on the isolated frog heart in which he found that the action of adrenaline was reversed after treatment with piperido-methyl-3-benzodioxane (933F), though the response to stimulation of sympathetic cardio-accelerator nerves was unchanged. Rosenblueth and Cannon (1936) have suggested that 933F may decrease the permeability of the cell membrane to adrenaline. The question arose as to whether the discriminative action of 933F was due to a difference in the nature of adrenaline and sympathin or to the possibility that sympathin is released intracellularly while adrenaline must reach the effector cell from outside the cell membrane. It seemed likely that light would be thrown on this problem by further study of the effect of 933F on the frog heart, testing sympathin secured from a second heart according to the method of Loewi. Unfortunately, we could not carry out this experiment because, as will be shown, we were unable to confirm Shen's observation that 933F discriminates between responses of the frog heart to adrenaline and to sympathetic stimulation. Our observations of the action of 933F on the amphibian heart led us to study also its action on the mammalian (cat) heart.

METHOD. The first series of experiments was carried out on 50 frog hearts from October, 1937, to February, 1938. The heart, together with a strip of tissue containing the right vago-sympathetic trunk, was removed from the frog and perfused according to the method of Straub. The nerve trunk was stimulated by means of a Harvard inductorium after perfusing the heart for a short time with atropine sulphate (1:5,000). Adrenaline (adrenalin of Parke, Davis & Company) was used in dilutions from 1:20,000 to 1:4,000,000. It was administered by adding a concentrated adrenaline solution to the perfusing fluid in the cannula until sufficient to result in the desired dilution. The drug 933F, in dilutions from 1:10,000 to 1:100,000, was administered by replacing the normal perfusing fluid with a fluid of similar composition containing the desired concentration of 933F. The Ringer's solution used as a perfusing fluid was adjusted with phosphate buffers to a pH ranging from 6.8 to 8.2, the latter pH being used in the majority of the experiments.

In the second series, 10 cats, anesthetized with dial (Ciba, 0.7 cc. per kgm. intraperitoneally), were used. A tracheal cannula was introduced and connected for artificial respiration. The adrenal glands were tied off. The heart was denervated by cutting the vagus trunks in the neck and removing the stellate ganglia and the upper thoracic sympathetic chains. The right cardio-accelerators were placed on shielded electrodes and stimulated for 10-second periods by means of a Harvard inductorium. Adrenaline (1 to 20 γ) and 933F (1 to 3.3 mgm. per kgm.) were injected into a femoral vein.

RESULTS. A. *The action of 933F on the frog heart.* The response to an initial treatment with 933F in 48 of the 50 experiments was a decrease in

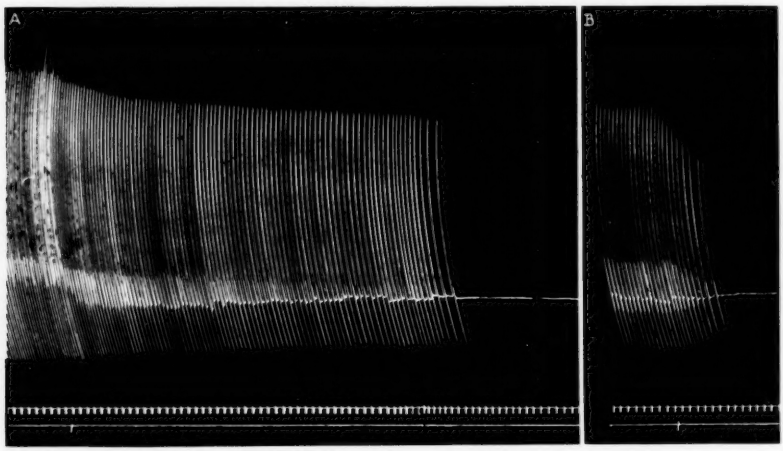

Fig. 1. Frog heart. In this and the succeeding records the time-signal marks 5-second intervals. *A*, at the signal perfusing fluid replaced by Ringer's solution (pH 8.2) containing 933F (1:10,000). Between *A* and *B*, the heart was washed several times with Ringer's solution. *B*, at the signal 933F (1:10,000) again administered.

rate or amplitude of contraction, or both. Often there was an initial stimulatory effect, particularly in the lower concentrations, but always the depressant effect became marked within 3 minutes. Subsequent administrations of 933F had a more rapid and intense depressant effect. The greater the concentration of 933F, the greater was its depressant action. At the higher concentrations complete stoppage of the heart occasionally occurred (fig. 1). In general, the more alkaline the perfusing fluid, the less depressant was the effect of 933F, but this pH difference was not marked and, compared with the differences due to concentration, was insignificant. In no case was the heart wholly unaffected by 933F.

B. *Responses to adrenaline and sympathetic stimulation after 933F.* The response to adrenaline after the administration of 933F was tested in 40 hearts, in 24 of which there were previous control observations with adrenaline. In 37 of the 40 experiments, the response was an increase in rate or amplitude, or both. In 3 experiments, in which there was a decrease in either rate or amplitude, the control observations with a similar amount of adrenaline before the administration of 933F had resulted in a similar depression. The concentrations of adrenaline used in these 3 cases were unusually high (1:50,000, 1:30,000, 1:20,000).

In 6 of the above experiments, the accelerator nerves also were stimulated before and after 933F. In all these experiments, the response after 933F was an increase in rate or amplitude, or both. It appeared, therefore, that 933F had the same effect on the response of the frog heart to adrenaline and to stimulation of the accelerator nerves (fig. 2).

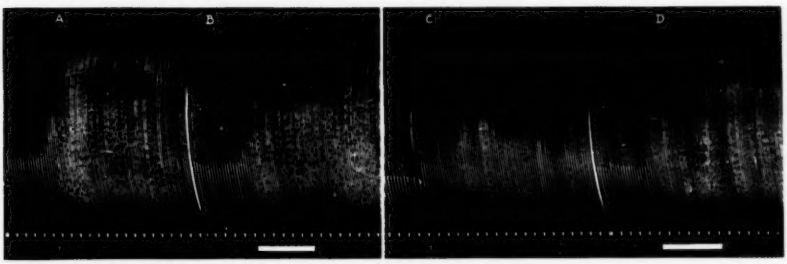

Fig. 2. Frog heart. Electrodes on right vago-sympathetic trunk. Heart washed with atropine (1:5,000) previously. *A*, adrenaline 0.25 γ added to perfusing fluid. The heart rate increased from 44 per min. to 60 per min. *B*, stimulation of vago-sympathetic trunk 30 sec. The heart rate increased from 44 per min. to 64 per min. Between *B* and *C*, 933F (1:70,000) administered. *C*, same as *A*. Heart rate increased from 38 per min. to 56 per min. *D*, same as *B*. Rate increased from 44 per min. to 60 per min.

In the majority of experiments the percentile increase in rate and amplitude of contraction in response to adrenaline and to sympathetic stimulation was greater after 933F than before its administration (cf. Rosenblueth and Simeone, 1934, for the desirability of comparison of percentile changes).

C. *The action of 933F on the cat heart.* The effect of 933F on the rate of the denervated heart was tested in 10 cats. In the majority there was a prompt, though slight, depression of the amplitude of contraction. The greater the amount of 933F injected, the greater was its depressant action. The heart rate was unaffected in the majority of cases.

D. *Response to adrenaline and sympathetic stimulation after 933F.* After 933F, the increase in rate resulting from adrenaline and from sympa-

thetic stimulation was less than the increase before 933F. The percentile acceleration of the heart rate was the same before and after 933F. The drug did not affect the response of the heart rate to adrenaline differently from its response to sympathetic stimulation (fig. 3).

As has been observed by others, the normal pressor effect of adrenaline on the blood pressure was either absent or reversed after 933F.

To test the activity of the 933F used, the contractions of the nictitating membrane were recorded in 2 of the experiments. In both cases adrenaline (in amounts up to 20 γ) which had previously resulted in a contraction of the membrane, had no effect on it after 933F. Stimulation of the cervical sympathetic nerve resulted in contractions of the membrane which were only slightly smaller after 933F than before its administration.

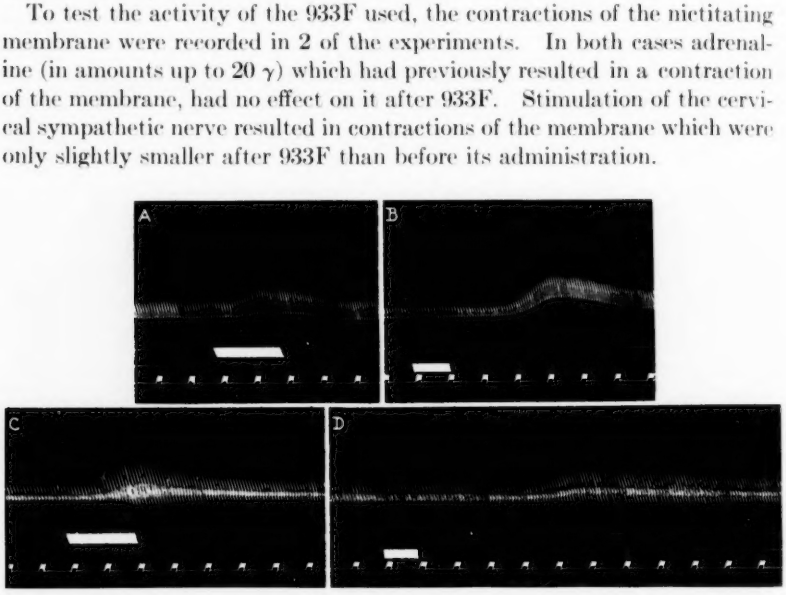

Fig. 3. Cat. Dial. Heart denervated, adrenal glands ligated. Electrodes on right cardio-accelerator nerve. All injections into right femoral vein. *A*, stimulation of accelerator nerve 10 sec. Rate increased from 138 per min. to 168 per min. *B*, adrenaline, 2 γ. Rate increased from 138 per min. to 168 per min. Between *B* and *C*, 933F (2.5 mgm. per kgm.) injected. *C*, same as *A*. Rate increased from 120 per min. to 138 per min. *D*, same a *B*. Rate increased from 120 per min. to 144 per min.

Discussion. Our results indicate that 933F has a depressant effect on the heart of the frog and the cat and that it has no differential effect on the response of this organ to adrenaline and to sympathetic stimulation. The reversal of the action of adrenaline on the frog heart treated with 933F reported by Shen (1937) might have been due either to the depressant effect of 933F on the heart or to the known toxic effect of adrenaline in high concentration. Our observations of the effect of 933F on the response of the frog heart to adrenaline are in agreement with those reported by Lissák (1938). The results of our experiments on the cat heart are in accord with deVleeschhouwer's (1935) incidental observation on the

innervated dog heart that after 933F adrenaline still caused tachycardia. It appears that the influence of 933F on the response of the heart to adrenaline is different from its influence on other sympathetic effectors (nictitating membrane, salivary glands, blood pressure, etc.).

To explain the different effects of 933F on the response of various tissues to adrenaline and to sympathetic stimulation, it has been suggested that 933F acts either by decreasing the permeability of the cell membrane to adrenaline (Rosenblueth and Cannon, *loc. cit.*) or by increasing the rate of destruction of adrenaline in the tissues (Morison and Lissák, 1938). Our observations are not readily explained by either of these suggestions.

It has been shown that the effect of sympathin on the nictitating membrane of the cat is reduced after 933F (Bacq, 1935). If 933F diminishes the response to adrenaline by reducing the cell permeability or by accelerating its destruction in the tissues, it might then be assumed to reduce the effect of sympathin by a similar mechanism. But such a hypothesis is hardly in agreement with the experiment of Luco and Lissák (1938), which demonstrated that sympathin is released into the aqueous humor of the cat's eye in equal amounts before and after 933F.

One must conclude that no adequate explanation of the action of 933F has yet been suggested.

CONCLUSIONS

1. The drug, 933F, has a depressant action on the isolated frog heart and on the denervated heart of the cat.

2. It has no differential effect on the response of the heart of the frog or of the cat to adrenaline and to sympathetic stimulation. These responses are only slightly modified by the drug.

We wish to express our gratitude to Dr. A. Rosenblueth and Dr. F. A. Simeone for their advice and encouragement.

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ON THE SPECIFICITY OF THROMBIN ACTION

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Alexander Schmidt's original theory of "fibrin ferment" as an agent of blood clotting (1), which for many years had been repeatedly attacked because of the newer findings in colloidal chemistry, is again coming to the fore and regaining recognition. In a previous communication, in which I described a method of serum gelation by ethyl alcohol (2) there arose the question as to the rôle of thrombin in the clotting of serum. It seemed possible that thrombin affected the globulins, or even perhaps all serum proteins. For blood clotting, the serum, since it contains no fibrinogen, plays a rôle totally different from that of the plasma. Moreover, the investigation on the second phase of the process of blood clotting was also based on the hypothesis that the transformation of fibrinogen into fibrin is (due to the thrombin) a ferment-catalytic phenomenon.

The analogy between the denaturation of proteins and the clotting of fibrinogen by the action of thrombin was demonstrated by Woehlisch (3). He states that thrombin represents a ferment which acts as a catalyzer for spontaneous denaturation of fibrinogen and terms this "denaturase." Woehlisch showed that the gel produced by either spontaneous or thrombin clotting of fibrinogen contained an identical protein in each instance. On the basis of this conception, he tried to identify the clotting by thrombin with the heat coagulation of fibrinogen. The so-called point of coagulation of proteins indicates that within the temperature limit in question, the denaturation occurs with much greater rapidity than below it. Chick and Martin (4) make responsible the extremely high temperature coefficient of proteins.

In the present work I shall follow essentially the point of view of Woehlisch (5). In 1923 Woehlisch (6) showed that heat denaturation, spontaneous denaturation, and the thrombin clotting of fibrinogen are essentially identical phenomena. Heat coagulation of proteins is strongly accelerated by the addition of ethyl alcohol, thus lowering the point of coagulation. Accordingly he demonstrated (which had already been indicated by Mellanby (7)) that by the addition of ethyl alcohol clotting by thrombin is greatly accelerated. If thrombin is merely a catalyst in the conversion of fibrinogen into fibrin it appears that the reason for the

failure of thrombin to coagulate the other globulins is the greater stability of these proteins as is indicated by their higher points of coagulation. On this basis if the point of coagulation of these globulins should be lowered by the addition of ethyl alcohol to that of fibrinogen (54°C.) then they should be coagulated in the presence of thrombin at this point. This Woehlisch showed does not take place. He concluded therefore that thrombin is strictly a specific catalyst.

In order to obtain further information on the specificity of the thrombin effect described by Woehlisch, I have tried to lower the coagulation point of serum far below the coagulation point of fibrinogen, namely, to room temperature. The method devised by me is of interest particularly because so little is understood of the phenomena involved in the coagulation of serum. The following are illustrative experiments.

EXPERIMENTAL. I used a dry powder of thrombin made according to the method of Mellanby-Bleibtreu (8). The crude thrombin solution was purified by a single acetone precipitation, a durable dry powder resulting. To obtain the active solution, the powder was first made into a paste by the addition of small quantities of physiological sodium chloride and then the salt solution added up to the desired volume. This suspension was placed in a shaking machine for 1 hour, and then filtered. In this manner thrombin solutions of 1 per cent, 2 per cent and 5 per cent were produced in physiological NaCl solution.

The effect of thrombin activity could be observed so that 0.04 ccm. citrate plasma, obtained from mixture of blood with isotonic (3.8 per cent) sodium citrate solution in proportion of 4:1, was made to clot within 20 seconds by the addition of 0.16 ccm. of the 1 per cent solution of dry thrombin at a room temperature of 24°C., and so that a gel with the transparency of glass was formed.

Human serum, obtained from spontaneously clotted blood, was diluted and for a short time (3 min.) kept at a room temperature of 24°C.: A, in proportion of 1:1 (0.1 ccm. serum each) with 0.1 ccm. each of (α) physiological NaCl solution; (β) 1 per cent thrombin solution; (γ) 2 per cent thrombin solution. B, in proportion 3:1 (0.15 ccm. serum each) with 0.05 ccm. each of (α) physiological NaCl solution; (β) 2 per cent thrombin solution.

Thereafter 0.2 ccm. of 96 per cent ethyl alcohol was added to each of the serum dilutions, and mixed well, so that the degree of gel formation could be measured and noted as follows: +++, complete; ++(+), almost complete; ++, strong; +(+), medium; +, weak; (+), beginning; −, none. Active as well as inactive serum (prepared by ½ hour heating in water bath up to 55–56°C.) was examined.

As can be seen from table 1, the serum gelation was in no way enhanced by the addition of freshly prepared thrombin solution. Our experiments

show that thrombin exercises no augmenting influence whatever on the clotting of either active or inactive serum, nor on such sera with greater protein content (B) at 24°C. or at 35.5°C.

It seemed of further interest to determine the influence of various thrombin concentrations on the clotting of serum.

At room temperature, 0.1 ccm. serum each, obtained from spontaneously clotted cattle blood was diluted with 0.1 ccm. each of a, 1 per cent thrombin

TABLE 1

Gelation of diluted human serum by addition of equal amounts of 96 per cent ethyl alcohol

MEASURED AFTER	SERUM DILUTION A = 1:1 WITH ADDITIONAL THROMBIN CONTENTS OF						Serum dilution B = 3:1 WITH ADDITIONAL THROMBIN CONTENTS OF			
	α 0		β 0.5 per cent		γ 1 per cent		α 0		β 0.5 per cent	
	a.	i.a.	a.	i.a.	a.	i.a.	a.	i.a.	a.	i.a.
At room temperature of 24°C.										
1 min.	−	−	−	−	−	−	−	−	−	−
3 min.	−	−	−	−	−	−	±	±	±	±
5 min.	−	−	−	−	−	−	(+)	(+)	(+)	(+)
15 min.	(+)	(+)	−	−	−	−	(+)	+	(+)	+
30 min.	(+)	(+)	±	±	−	−	+	+	+	+
45 min.	(+)	(+)	±	±	±	±	+	+(+)	+	+(+)
2 hrs.	+	+	(+)	(+)	(+)	(+)	+(+)	++	+(+)	++
24 hrs.	+	+	(+)	(+)	(+)	(+)	++	++	+(+)	++
At incubator temperature of 35.5°C.										
1 min.	−	−	−	−	−	−	−	−	−	−
3 min.	−	−	−	−	−	−	(+)	+	(+)	+
5 min.	−	±	−	±	±	±	+	+(+)	+	+(+)
15 min.	(+)	+	(+)	+	(+)	+	++	++(+)	++	++(+)
30 min.	+	+	(+)	+	(+)	+	++(+)	++(+)	++	++(+)
45 min.	+	+(+)	+	+	+	+(+)	++(+)	+++	++	++(+)
2 hrs.	+	+(+)	+	+(+)	+	+(+)	+++	+++	+++	+++
24 hrs.	++	++	+(+)	++	+(+)	++	+++	+++	+++	+++

a = active serum; i.a. = inactive serum.

solution; b, 2 per cent thrombin solution; c, 5 per cent thrombin solution; d, physiological NaCl solution. By addition of 96 per cent ethyl alcohol, the final volumes of 0.4 ccm. and 0.5 ccm. were obtained. The gelation was done at a room temperature of 24°C. and an incubator temperature of 35.5°C. After 5 minutes, 30 minutes, 4 hours, and 3 days, respectively, the degree of gel formation was measured and noted in the above described manner.

The result of this experiment is equally striking. Table 2 shows first that coagulation of animal serum is not accelerated by thrombin; second, that a higher alcohol proportion of the mixture (57.6 per cent instead of 48 per cent) raises the degree of gel formation, but does in no way show an increased thrombin effect. Finally, it is noteworthy that even with the addition of the strongest thrombin solution (additional content of 1.25 per cent thrombin) no augmentation of the degree of serum gelation occurred. It may be mentioned in this connection that the original proportion of thrombin in the serum had not been ascertained. The results, as shown in tables 1 and 2, were confirmed in a series of further experiments. Various human sera, fresh and those aged by storing, also sera of pigs and

TABLE 2

Gelation of cattle serum (0.1 ccm.) diluted with equal parts of physiological NaCl solution of thrombin in varying percentages

MEASURED AFTER	*a* 1 PER CENT THROMBIN		*b* 2 PER CENT THROMBIN		*c* 5 PER CENT THROMBIN		*d* 0	
	by 96 per cent ethyl alcohol							
	0.2 ccm.	0.3 ccm.	0.2 ccm.	0.3 ccm.	0.2 ccm.	0.3 ccm.	0.2 ccm.	0.3 ccm.
At room temperature of 24°C.								
5 min.	−	−	−	−	−	−	±	±
30 min.	−	−	−	−	±	−	±	(+)
4 hrs.	+(+)	++	+(+)	++	+(+)	+(+)	+(+)	++
3 days	+(+)	++	+(+)	++	+(+)	++	+(+)	++(+)
At incubator temperature of 35.5°C.								
5 min.	±	−	−	−	−	−	±	±
30 min.	+	+	+	+	+	+	+	+(+)
4 hrs.	+(+)	++(+)	+(+)	++	+(+)	++	+(+)	++(+)
3 days	+(+)	++(+)	+(+)	++(+)	+(+)	++(+)	+(+)	++(+)

horses, were examined. Incidentally, as may also be seen from the tables, thrombin showed a temporary inhibitory effect upon the gelation of serum. However, the differences in the degree of gel formation were so trifling that they more or less remained in the range of optical error. It is impossible, therefore, to conclude that thrombin has an inhibitory effect on the clotting of serum.

Discussion. Our experiments have shown that thrombin does not accelerate the coagulation of serum. What may be concluded is that thrombin has a strictly specific effect on the conversion of fibrinogen to fibrin. Our experiments, however, do not permit any definite conclusions as to the exact nature of the mechanism operating herein. The fact that hitherto all hypotheses that were offered to replace the ferment theory are

lacking in experimental proof (9) rather seems to be in favor of the assumption that thrombin functions as a catalyzer. The question of the ferment nature must remain undecided as long as evidence is offered to show that in fibrin formation thrombin is consumed and becomes a constituent of fibrin.

It might be suggested that thrombin which is added to serum is, due to adsorption, bound either to serum protein, or in some at present unknown manner so that a thrombin effect becomes impossible. This also raises the question of inactivation of thrombin. According to Morawitz (10), the spontaneous reduction of activity of serum thrombin is due to the transformation of this thrombin into a neutral modification, metathrombin, which, according to Howell (11), is a combination of thrombin with the so-called anti-thrombin of the serum. On the other hand, Gratia (12) has pointed out that by the term anti-thrombin is understood the function of preventing the formation of thrombin. Various investigators have assumed and to some extent proved the existence of other substances in the organism that are apt to check clotting. These inhibitory substances, according to Howell and Holt (13), are among others regarded as pro-anti-thrombin and anti-prothrombin; the latter is present in the circulating blood. The undoubted neutralization of thrombin in serum makes the existence of a proper anti-thrombin highly probable. According to Mellanby (l.c.7), however, anti-thrombin is extremely alcohol sensitive. In the preparation of thrombin according to the method of Alexander Schmidt which is based upon the action of alcohol on serum, anti-thrombin is completely destroyed. It is important therefore to inquire about the stability of thrombin. According to Woehlisch (l.c.5), aqueous solutions of thrombin, obtained by the method of Mellanby and Bleibtreu, may remain active for weeks. Several experiments, like those of Blaizot (14) and Hiruma (15), have demonstrated that thrombin becomes inactive in the serum only after standing for hours. Thus, in our experiments, the anti-thrombin effect of several minutes could but hardly or little influence the activity of thrombin, especially since the addition of alcohol both destroys the anti-thrombin and increases the thrombin effect.

SUMMARY

1. The influence of thrombin on the clotting of various sera (human, cattle, horse, and pig) was examined by the method of serum gelation by ethyl alcohol.

2. Both active and inactive sera, fresh and those aged by storing, were examined with increased alcohol as well as protein content of the serum-alcohol mixture.

3. Woehlisch's observation that globulins, after lowering (with alcohol) their point of coagulation to that of fibrinogen at 54°C., are not affected by

thrombin, applies also to all proteins in the serum. We extended this by lowering the coagulation point of the serum to 35.5 and 24°C.

4. Throughout thrombin shows no augmentation of serum coagulation. From this it may be concluded that thrombin exercises a strictly specific effect, probably as catalyzer, on the conversion of fibrinogen to fibrin.

5. In connection with the above-mentioned problems, the question of the inactivation of thrombin is briefly discussed.

I wish to thank Professor R. Stachelin, University of Basle, for making available to me the facilities of his laboratory and Professor E. Woehlisch, University of Wuerzburg, for supplying me with his preparation of thrombin.

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ON THE EFFECTS OF ACUTELY RAISED INTRACRANIAL PRESSURE ON DIURESIS IN THE DOG

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With but two exceptions (1, 2), there has been little reference in the literature, either clinical or experimental, to the effects of raised intracranial pressure (I.C.P.) on urine excretion. In this laboratory, however, more than two years ago, Libet and Yesinik (unpublished) observed that an acute elevation of the intracranial pressure in the dog caused a diminution or cessation of urine flow, despite the accompanying great rise of the carotid blood pressure. It was felt that these observations could bear further investigation since the respiration ceased shortly after marked elevation of the intracranial pressure and no attempt was made to maintain an oxygenated condition of the blood by artificial respiration.

METHODS AND PROCEDURE. In our early acute experiments, performed on dogs under barbital-Na anesthesia (intravenous—250–290 mgm./kgm. of body weight), diuresis was induced by a single intravenous injection of 200 cc. of 5 per cent glucose solution. Urine was collected via a glass bladder cannula[2] in three minute intervals before, during, and after an abrupt increase of intracranial pressure (also for three minutes) maintained at a level well above the systemic blood pressure, as measured, in the usual way, from the common carotid artery. Intracranial pressure was raised by a modification of the method of Cushing (3), the pressure being elevated by a 0.9 per cent sodium chloride pressure bottle system, which communicated both with the cranial cavity, through a metal cannula screwed into a trephined hole in the midparietal portion of the calvarium, and with a mercury manometer which recorded the intracranial pressure. The rate and relative amplitude of the respiratory movements were recorded simultaneously by the usual pneumograph-tambour method. Atropine sulphate (0.5 cc. of a 0.1 per cent solution/kgm. of body weight) was administered, subcutaneously, shortly before the start of trials, to eliminate cardio-inhibitory effects. As a consequence, the increased intracranial pressure produced maximum blood pressure rises.

In these early studies, urine collections were made after the single in-

[1] The present investigation was aided in part by a grant to the University of Chicago from the Rockefeller Foundation.

[2] Cannula designed by Libet and Yesinik. (In Press: J. Lab. and Clin. Med.)

travenous injections of the 5 per cent glucose solution (5 per cent sucrose was also used in later experiments), and the intracranial pressure was then raised periodically in the course of the waning diuresis. However, when superimposed upon the otherwise declining curve of diuresis, the observed diminutions in urine volume during the raised intracranial pressure were not so striking as to be indubitably ascribed to the elevated intracranial pressure and not to some coincidental fluctuation in the normal rate of falling off of the diuretic response. We, therefore, devised a method whereby a constant and significant diuresis (in the face of the barbital depression (4)) was induced so that any changes, in this relatively constant diuresis, during raised intracranial pressure would be detected readily and incontrovertibly. To achieve this end, a continuous intravenous infusion of 5 per cent glucose (later 5 per cent sucrose) solution was given (2.5 cc./min.) immediately after the initial injection of 200 cc. (5 per cent glucose solution). A fairly constant diuresis was obtained. With this procedure, abruptly raising the intracranial pressure and holding it above the systemic blood pressure level for a period of three minutes produced diminutions in urinary volume which could now be directly correlated with raised intracranial pressure and also with general carotid blood pressure changes resulting from the latter.

However, in this preliminary work, it was soon observed that during the elevated intracranial pressure and respiratory standstill (3, 5, 6) the blood in the carotid cannula became almost black—indicating profound anoxemia. Experimental results in these cases were discarded since anoxia, in itself, causes marked diminution in urine excretion (7). Artificial respiration (adjusted as closely as possible to the dog's normal rate and depth) was administered promptly on cessation of the respiration following elevation of the intracranial pressure. The red color of the blood was thereafter used as an index of successful ventilation and oxygenation of the blood. The rate of urinary excretion and the state of the blood pressure were not affected by the degree (rate and amplitude) of the artificial respiration administered in these experiments.

Results and discussion. Under these experimental conditions, any sustained appreciable elevation of the intracranial pressure above the carotid blood pressure (twenty-six trials on nine barbitalized dogs) effected significant diminutions in the volume of urine excreted in three minute intervals. Figure 1 records graphically a typical experiment showing diminutions of urine excretion, ranging from forty to sixty-eight percent, during periods of raised intracranial pressure. Table 1 gives the averaged values obtained in all the dogs of this series. Other experiments showed even greater decreases (than represented in fig. 1) amounting often to an absolute cessation of urine excretion, despite the great increase in systemic blood pressure present during raised intracranial pressure (3, 5, 6).

Claude Bernard (quoted by Bieter, 8) first demonstrated that stimulation of the splanchnics causes diminution of urine flow and that section of these nerves results in polyuria. Many investigators have related changes in urinary output to neural influences. By stimulating the central ends of divided sensory nerves, Bradford (quoted by Bieter, 8) confirmed Cohnheim and Roy in finding reflex vasoconstriction of the renal vessels. This, and similar work (9, 10, 12, 13, 14, 15, 16, 17), proved that as far as blood vessel effects are concerned, the kidney is well supplied with vasoconstrictor nerves and, to a lesser extent, with some vasodilator fibers.

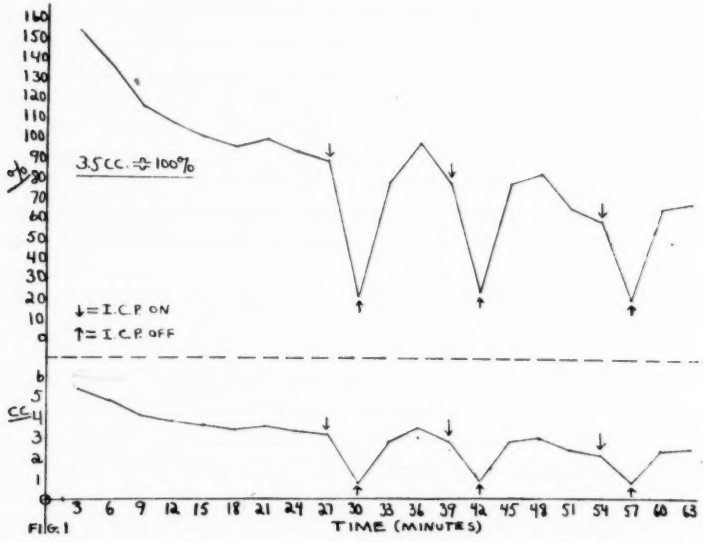

Fig. 1. Graph showing volume and percentile variations of urine excretion during control periods and periods of acutely raised intracranial pressure. I.C.P. = acutely raised intracranial pressure usually some 50 mm. Hg or more above the carotid blood pressure.

The decreased urine volume observed during acutely raised intracranial pressure and the ensuing high blood pressure, might be the result of renal anemia due to intense vasoconstriction of the renal arterioles. Accordingly, denervation of one kidney was performed, aseptically, so that the effects of acutely raised intracranial pressure on the normal and denervated kidney could be compared simultaneously in the same animal.

The denervations of a single kidney were performed *two or more weeks before experimentation.* The approach to the kidney was retroperitoneal and the denervation was affected by vascular stripping (9, 10) supplemented

by painting the renal vessels with phenol (and then neutralizing the phenol with 70 per cent alcohol). This method probably does not effect an absolutely complete denervation (only removal and then replacement of the kidney can assure that), but the denervation, so produced, is extensive enough for our purpose.

The subsequent experimental procedure in the acute experimentation was the same as that originally employed on the normal animals, with one exception, namely, the urine was not collected by a bladder cannula but by special ureteral cannulization (11), since it was essential to collect the urine separately from each kidney.

Twenty-two trials in six dogs showed that, whereas the normal control kidney yielded the characteristic diminution of urinary volume during the

TABLE 1

Intact kidney animals

Averages of urine excretion in three minute intervals

DOG NO.	NO. OF TRIALS (I.C.P.)	EXCRETION BEFORE I.C.P.	EXCRETION DURING I.C.P.	EXCRETION AFTER I.C.P.
		cc.	cc.	cc.
1	1	2.30	0.30	2.50
2	4	1.93	1.05	1.89
3	2	2.66	0.67	1.52
4	3	4.30	0.72	1.03
5	1	3.50	1.03	2.70
6	2	1.45	0.99	1.02
7	4	3.91	0.83	2.48
8	4	4.25	1.82	4.30
9	5	4.21	1.30	3.19
Grand average		3.16	0.96	2.29

periods of acutely raised intracranial pressure, the denervated kidney excreted, for these same periods, only slightly less, the same amount, or even a greater quantity of urine than during its normal periods. A typical experiment (fig. 2) is representative of all the results on this phase of the work. Table 2 gives the averaged values obtained in this series; and figure 3 shows graphically the grand average percentile difference in urine excretion between the denervated and normal kidneys during control periods and periods of acutely raised intracranial pressure (twenty-two trials) of the same series.

Thus, the urinary diminution observed in acutely raised intracranial pressure in normal dogs is apparently dependent upon the integrity of the renal (vasoconstrictor) innervation.

It has long been known that stimulation of the peripheral ends of cut

splanchnic nerves leads to diminished renal output, an effect which has been correlated with a postulated renal vasoconstriction as recorded by different criteria (12, 13, 14). Bieter (8), studying the effects of nerve stimulation on the renal capillaries in the frog, reported that stimulating the peripheral end of the cut splanchnic, or central end of the cut sciatic or vagus nerve resulted in cessation of blood flow through many of the glomeruli so that the number of patent glomeruli was reduced about fifty per cent. With discontinuance of the stimulation, the inactive glomeruli resumed function.

A similar reduction of glomerular blood supply has been postulated in

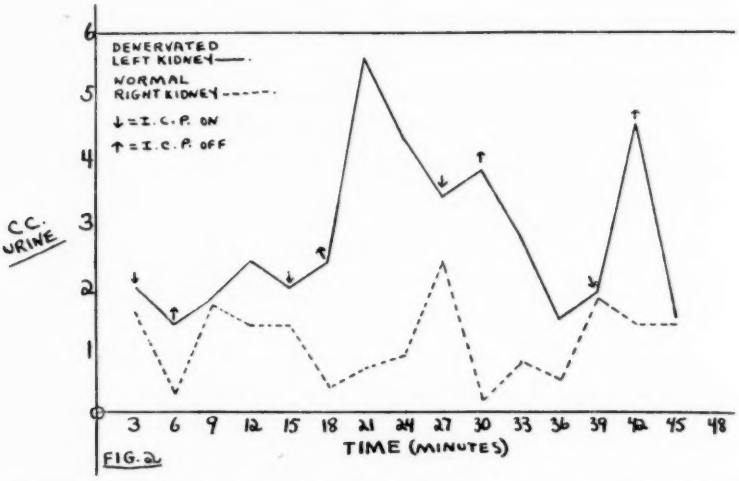

Fig. 2. Graph showing the consistently higher urinary volume excreted by the denervated kidney in all periods; also the opposite reactions of the normal and denervated kidneys to the results following acutely raised intracranial pressure.

the mammalian kidney's response to numerous irritating conditions. For example, the reflex anuria seen clinically probably results from such a mechanism. Loucks and Scott (quoted by Bieter, 8) reported anuria after pulling on the kidneys, stretching muscles, or cutting the skin in dogs. Presumably, this effect results from a renal vasoconstriction produced reflexly. Bieter (8), (frog), observed that placing an artery clamp on a cocainized ureter or on the ureter of a kidney whose splanchnic nerve supply had been sectioned did *not* produce a decrease in the number of active glomeruli comparable with the results obtained by such a maneuver with intact splanchnics and an uncocainized ureter. The decrease in active glomeruli is possibly due to reflex closure of the afferent glomerular

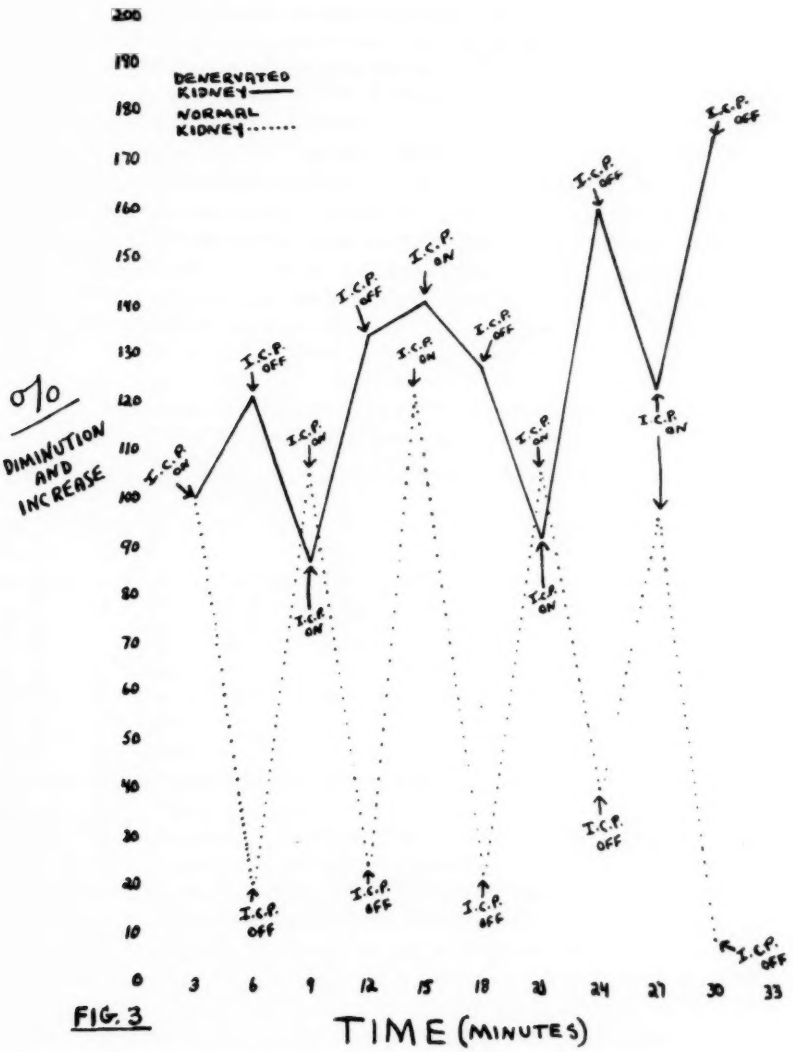

FIG. 3

Fig. 3. Composite curves for denervated (left) and normal (right) kidneys showing average urine collections expressed in per cent. These curves represent the values given in table 2 for the six dogs in this series.

arterioles. Vasomotor changes of the vasoconstrictor type due to central discharges or induced by stimulation of afferent nerves may and can reduce the volume of the urinary output even when an increased general blood pressure (as in our experiments) would otherwise increase the urine output.

Much evidence supporting this point of view has been accumulated from another type of experimentation; namely, observation of the effect of denervation upon the mammalian kidney. Many investigators have reported that increased blood flow, hypertrophy, and diuresis occur in the denervated kidney (10, 12, 14). All authors agree that denervation causes a marked increase in the vascularity of the kidney with obvious dilatation of the renal arterioles and capillaries (15, 16, 9, 17). Frequently, accompanying these changes, there occurs a diuresis that may persist for a few weeks or a few months (18, 19, 20, 21). These observations are substanti-

TABLE 2

Left (L) kidney denervated, Right (R) kidney normal

Averages of three minute urine collections

DOG NO.	NO. OF TRIALS (I.C.P.)	EXCRETION BEFORE I.C.P.		EXCRETION DURING I.C.P.		EXCRETION AFTER I.C.P.	
		L.	R.	L.	R.	L.	R.
		cc.	cc.	cc.	cc.	cc.	cc.
1*	3	1.40	2.50	2.57	0.70	1.35	3.00
2	3	1.90	1.66	2.60	0.40	2.84	2.40
3	4	1.72	1.24	3.00	0.57	2.57	1.24
4	3	3.45	3.32	4.37	0.83	6.00	3.70
5	5	2.40	2.02	2.80	0.20	2.56	1.87
6	4	2.12	1.84	1.85	0.40	1.48	1.37
Grand average.....		2.16	2.09	2.86	0.51	2.80	2.26

* The one kidney was denervated a few minutes before recording urinary volum e

ated by Bieter's demonstration in the frog (8) that section of all the visible sympathetic trunks running toward the abdominal aorta on one side produces an increase in the number of active glomeruli on the homolateral side. Thus it may be concluded that the immediate effects of splanchnic nerve section are an increase in the number of active glomeruli and an increase in urinary volume, unless section of the splanchnics also interferes with tubular absorption of water.

Nevertheless, some investigators have claimed that the renal nerves play no rôle in the excretion of water. Smith, in his monograph (22), discusses some of the conflicting data on this question. He compares the earlier work of Marshall and Kolls (13) with that of Bykow and Alexejew-Berkmann (23) and of Klisiecki, Pickford, Rothschild, and Verney (24). The former investigators observed in dogs (under paraldehyde anesthesia)

that cutting the spanchnics or stripping off the renal plexus resulted in the excretion from the operated kidney of a urine which was greater in volume and more dilute in chloride than that of the normal control. This result, they concluded in a series of later papers, was due solely to changes in the blood flow and the pressure relations consequent to denervation. Smith agrees that many others have observed this diuresis in denervated kidneys. On the other hand, both of the latter groups (23, 24) of investigators reported that in unanesthetized dogs, the urine flow in the normal and denervated kidneys was about the same and that both kidneys were affected equally by conditioned reflex inhibition of water excretion, by extinction of the conditioned reflex (23) and by exercise and afferent stimulation. These investigators concluded, therefore, that the renal nerves play no rôle in the excretion of water.

Smith (22), in reviewing these conflicting views, contends that urine flows, in the work of Marshall and Kolls (13), were too low and urine too concentrated to justify their conclusions. Our work, however, answers the criticism which Smith makes of these early workers; for by the method of continuous sucrose (or glucose) infusion we have been able to study the function of normal and denervated kidneys in dogs under barbital-Na anesthesia with urinary volumes about equal to that observed in the normal dog. Although the diuresis induced is not equal in intensity to that observed in the unanesthetized animal, the volumes observed are sufficiently great to permit unequivocal recognition of altered kidney output in each of the two kidneys. Under these conditions, it can be seen (refer to table 2, and figs. 2 and 3) that in all five of five previously operated animals, urine volumes, in the control periods and after raising intracranial pressure, ran consistently higher in the denervated than in the innervated kidney. This evidence supports the existence of a "denervation diuresis."

In addition, our data give evidence of an alteration in the volume of excreted urine, mediated through the renal nerves. The diminution in urinary output observed during acutely raised intracranial pressure is most probably due to renal vasoconstriction resulting from profound asphyxial stimulation of the vasomotor center. This vasoconstriction is of such magnitude as to decrease the renal blood flow sufficiently to cause a lowered renal output despite the concomitant great rise in blood pressure.

We have obtained, during acutely raised intracranial pressure, blood pressures ranging from 230 to 360 mm. Hg without a drop of urine being excreted by the normal (innervated) kidneys, whereas the denervated kidneys maintained their normal output or excreted even more. We have also found that, in those experiments with raised intracranial pressure in which we "split" the three minute collections into three one minute intervals, any urine, appearing on the innervated side, occurs during the first minute when the blood pressure has not, as yet, risen to great heights;

that is, when the vasoconstriction is not, as yet, maximal; and, in all cases where the blood pressure rise is almost immediate, no excretion occurs in the first minute. Ofttimes, however, when beginning failure of the vasomotor center occurs in the third minute (followed by rather steep drops in the blood pressure), some urine appears on the innervated side; this urine probably indicates partial relaxation (dilatation) of the renal arterioles.

We have no explanation as to the rôle of the very abundant number of vasoconstrictor fibers in the nerves of the normal kidney, during the life of an individual with its calm periods and periods of emergency. However, the diuresis, following renal denervation, indicates that these nerves may be normally in a state of tonic activity. The transient character of this denervation diuresis observed by some investigators is perhaps due to the gradual establishment of independent tonus by the afferent arterioles going to the glomeruli resulting eventually in urinary volumes approaching the normal.[3]

Further studies of the rôle renal nerves play in altering kidney function under physiologic conditions in normal unanesthetized animals are in progress.

SUMMARY

Acutely raised intracranial pressure causes a marked diminution and often cessation of the diuretic response in the dog despite the accompanying great rise in systemic blood pressure.

In dogs in which one kidney was denervated, similar diminutions in the urine flow were observed in the normal kidney, whereas maintenance or augmentation of the diuretic response was observed in the denervated kidney, during periods of acutely raised intracranial pressure.

It has been shown that the denervated kidney, in dogs under barbital-Na anesthesia, excretes a greater volume of urine than the normal kidney.

The abolition or lower rate of urinary excretion in normally innervated kidneys during raised intracranial pressure is due to renal vasoconstriction mediated through the vasomotor center, the constriction being of such magnitude as to decrease the renal blood flow sufficiently to cause a lowered renal output, despite the great rise in blood pressure. In fact, the great rise in general arterial blood pressure under these conditions is due in part to the intense vasoconstriction of the abdominal viscera including the kidneys.

The authors wish to acknowledge the welcome criticism and advice of Dr. Arno B. Luckhardt.

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BILE PIGMENT AND HEMOGLOBIN INTERRELATION IN ANEMIC DOGS

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When hemoglobin (18) is destroyed within the body the pigment radicle is quantitatively transformed into bilirubin and eliminated in the bile as a waste product of pigment metabolism. Experiments in this laboratory and others performed by Blankenhorn (1) and by Bollman, Sheard and Mann (2) clearly show that bile pigments are not reabsorbed from the intestinal tract. Bilirubin is reduced in the intestine to urobilin and some of this is absorbed as has been shown by McMaster and Elman (13) but there is no evidence that this urobilin bears any relation to the output of bilirubin.

Since the pigment radicle comprises 4 per cent of the hemoglobin molecule it has been customary to use the ratio 40 mgm. of bilirubin = 1 gram of destroyed hemoglobin. However, the formula of bilirubin is now better established and it is considered to have the molecular weight 584. Hemin has a molecular weight 652 and 1 gram of hemoglobin contains 3.35 mgm. of iron. On the basis of these data when 1 gram of hemoglobin is destroyed there should result 34.9 mgm. of bilirubin. We have used in this report the figure of 35 mgm. as equivalent to 1 gram of hemoglobin.

Previously we reported (6) that upon the injection of laked hemoglobin into anemic bile fistula dogs it was conserved with the production of an equivalent amount of new formed hemoglobin and at the same time there was an excess excretion of bile pigment amounting to 75 per cent of the injected hemoglobin. This was an unexpected reaction and has important bearings on the understanding of pigment metabolism. Since this reaction is of such fundamental importance we considered it advisable to repeat the experiments. The data in this report show conclusively that upon the injection of laked hemoglobin there is practically 100 per cent recovery of it as new formed hemoglobin in red cells and concurrently that there is quantitative elimination of the pigment radicle of the katabolized hemoglobin as bilirubin.

METHODS. Two types of bile fistula dogs were used, the gall bladder renal fistula of Kapsinow, Engle and Harvey (9) and the sterile closed bag

fistula devised by Rous and McMaster (16) as modified by Smith, Groth and Whipple (17).

The renal fistula dogs are kept in galvanized iron metabolism cages, and the urine bile mixture is collected over 24 hour periods. Chloroform, 5 cc., is put in the collection bottle as a preservative. The dogs are given 200 cc. of water by stomach tube 3 hours before the collection of the 24 hour samples. This water causes the dogs to void regularly before the collections of the urine and thus makes for more uniformity in the daily amounts of bile pigment. The bag fistula dogs are dressed once a day with collections of the 24 hour accumulation of sterile bile.

All of the dogs are fed the salmon bread diet both because its hemoglobin building properties have been thoroughly studied, but also since it is a diet low in fat and rich in carbohydrate and therefore well tolerated by these bile deprived dogs. It is in itself a complete ration which will maintain dogs in health and normal activity indefinitely. It contains wheat flour, potato starch, canned salmon, sugar, cod liver oil, tomatoes, yeast, and a salt mixture. Its preparation has been previously described (19).

In order to prevent the intoxications (7, 5) that result from complete bile deprivation the dogs are given dog bile or ox bile, 50 cc., on the food daily.

Methods for the determination of bile pigments (11) in both urine and bile have been described in detail in a previous publication.

The blood hemoglobin level is determined following the method of Robscheit (14) and blood volume by the brilliant vital red technique. In all tables hemoglobin values are given in per cent—the standard 100 per cent = 13.8 grams of hemoglobin per 100 cc. The dogs are made anemic by bleeding and the details relating to this procedure have been described by Whipple and Robscheit-Robbins (19). The formula for estimating the total hemoglobin removed by bleeding is given in a previous report (20). The dog's base line production of hemoglobin on the salmon bread diet is established and this amount is subtracted from the total amount of hemoglobin removed during the experimental period. Care was taken to exhaust the stores of hemoglobin building materials which the body can call out in the early weeks of the severe anemia. If such reserve stores are not depleted erroneous results are bound to be obtained.

In preparing hemoglobin for injection, freshly drawn blood is centrifugalized for 35 minutes, the plasma sucked off, and then the packed cells are washed twice with physiological saline. Two volumes of distilled water are added to the washed packed red cells, which are then laked, centrifugalized and the hemoglobin solution filtered through gauze. One cubic centimeter of the hemoglobin solution is diluted with 99 cc. of 0.1 normal hydrochloric acid and allowed to stand 3 hours, after which the hemoglobin value is estimated using a 1 per cent solution of acid hematin

(14) as standard. This solution of hemoglobin is injected into the jugular vein in amounts equivalent to 2 or 3 grams hemoglobin. Amounts above this cannot be used as the renal threshold would be exceeded and some would consequently be lost in the urine (12).

EXPERIMENTAL OBSERVATIONS. The experimental data obtained from these three dogs are quite similar. In each instance the introduced hemoglobin contributes, as has been shown before, to the rebuilding of new hemoglobin in anemia with 90 to 100 per cent conservation. At the same time there is a large excess of bile pigment excreted above the control level and this obviously comes from the katabolism of the introduced hemoglobin.

In table 1 (dog 35–15) a satisfactory experiment is given in detail. The dog was a mongrel female hound and the *renal fistula* was made on November 12, 1936. After the operation it established its weight at 14.5 kgm. which is similar to the weight during this experiment 1 year and 3 months later. The basal ration was 250 grams salmon bread, 75 grams salmon, 30 grams "Klim" mixed with water into a mash. Either dog or ox bile, 50 cc. daily, was fed by stomach tube or on the food. On December 9, 1937, bleeding was started and the reserve stores of hemoglobin building materials were exhausted before the experiment was started. After the anemia level of 50 per cent was obtained it was established that the dog's basal output on the salmon bread was 4.3 grams of hemoglobin a week. During the injection period of 2 weeks, 42 grams of hemoglobin were injected in 3 gram amounts daily. There was no gross evidence of hemoglobin in the urine. As the result of the introduced hemoglobin, 38 grams of new hemoglobin were formed, or a recovery of 91 per cent. At the same time 1295 mgm. of bile pigment were excreted in excess of the control level. As 1 gram hemoglobin is equivalent to 35 mgm. of bile pigment, we should expect 1470 mgm. to be formed from the 42 grams of injected hemoglobin. We actually recovered 88 per cent of the expected amount. The daily average bile pigment during control periods was 61 mgm. Plasma volume and weight remained constant.

It is evident that the injected hemoglobin has been utilized to form new hemoglobin and yet at the same time we recover bile pigment which corresponds to 88 per cent of the injected pigment radicle. Presumably the excess bile pigment results from the splitting off of the pyrrol aggregate and the increase in hemoblogin is due to the retained iron and possibly globin fractions of the injected hemoglobin combined with the newly formed pigment radicle.

The results obtained from a *bag fistula* dog are given in table 2. Dog 37-75 was a female Scottie and the bag fistula was made on December 15, 1937. It was fed a basal diet of 200 grams salmon bread, 75 grams salmon, and 30 grams "Klim" with dog bile, 50 cc., added to the food. Bleeding

was started on December 21, 1937, and the reserve stores were depleted by repeated bleeding and finally the basal output of hemoglobin became 2.2 grams per week on an average. During the experimental period 31 grams of hemoglobin were injected and 27.2 grams of new hemoglobin were formed and recovered by bleeding. This is equal to 88 per cent of the amount

TABLE 1

Bile pigment and hemoglobin recovery after hemoglobin injection

Dog 35-15—Renal fistula, anemic

DATE	BILE PIGMENT OUTPUT 24 HOURS	HEMOGLOBIN INJECTION 24 HOURS	HEMOGLOBIN RECOVERY BY BLEEDING	BLOOD HEMOGLOBIN LEVEL	BLOOD VOLUME	URINE BILE MIXTURE	WEIGHT
	mgm.	*gm.*	*gm.*	*per cent*	*cc.*	*cc.*	*kgm.*
Feb. 17	70					800	13.8
18	48					620	
19	78		6.6	57		930	
20	38					430	
21	63					1,020	
22	59	3	1.5	48	1,150	1,085	
23	143	3				700	14.0
24	144	3				960	
25	137	3				890	
26	154	3				1,120	
27	149	3				820	
28	161	3				1,160	
Mar. 1	150	3	13.0	67		1,060	
2	155	3				600	14.0
3	157	3	15.4	57		585	
4	155	3				520	
5	176	3				935	
6	140	3				550	
7	144	3				650	
8	184		15.6	60	1,170	1,535	
9	80					890	14.0
10	47		0.8	51		400	
11	82					980	
12	59					550	
13	44					500	
15	73		5.6	52	1,129	880	14.2
17	50		0.8	53		540	

injected. Over a control period of 20 days the average daily output of bile pigments was 42 mgm. As the result of the hemoglobin injections, 965 mgm. of bile pigment were excreted in excess. This is a recovery of 89 per cent, as 1085 mgm. represents the theoretical amount contained in the introduced hemoglobin. Plasma volume and weight remain constant.

Table 3 (dog 36–108) shows the data from another bag fistula dog. The fistula was established on December 8, 1937, and the basal diet was 300 grams salmon bread, 75 grams salmon, and 30 grams "Klim" with dog bile, 25 cc., added to the food. Bleeding was commenced on December 18 and the basal output of hemoglobin was determined as being 5.3 grams a week on an average. During control periods of 10 days before and after

TABLE 2

Bile pigment and hemoglobin recovery after hemoglobin injection

Dog 37-75—Bag fistula, anemic

DATE	BILE PIGMENT OUTPUT 24 HOURS	HEMOGLOBIN INJECTION 24 HOURS	HEMOGLOBIN RECOVERY BY BLEEDING	BLOOD HEMOGLOBIN LEVEL	BLOOD VOLUME	BILE VOLUME	WEIGHT
	mgm.	*gm.*	*gm.*	*per cent*	*cc.*	*cc.*	*kgm.*
Feb. 18	39				833	124	
19	42		0.9	55		118	
20	40					144	
21	44					126	
22	42	3	1.5	50	892	138	11.5
23	115	3				112	
24	143	3				142	
25	129	2				152	
26	129	2				122	
27	98	2				126	
28	101	2				132	
Mar. 1	98	2	11.1	67		130	
2	106	2				124	
3	120	2	8.6	58		130	
4	113	2				122	
5	82	2				98	
6	112	2				182	
7	75	2				124	
8	112		12.3	56	815	186	11.2
9	63					132	
10	55		0.8	51		134	
11	44					160	
12	54					140	
15			0.8	54			

the experimental period the daily average bile pigment excretion was 63 mgm. As the result of the injection of 42 grams of hemoglobin 1414 mgm. of bile pigment were excreted in excess, or 96 per cent of the expected yield of 1470 mgm. By bleeding 41 grams of new hemoglobin in red cells were recovered, or 98 per cent of that injected.

A repeat experiment on this dog was performed and the results are tabulated in table 4. During the 10 day control periods before and after the

injection period the bile pigments averaged 74 mgm. a day. It is to be noticed that the output is greater in the 4 day after period than in the fore period. This is due to a small hematoma that formed on the next to the last day of the injection period. The experiment is, therefore, complicated but the results are comparable with the previous ones. Over the 2 week

TABLE 3

Bile pigment and hemoglobin recovery after hemoglobin injection

Dog 36-108—Bag fistula, anemic

DATE	BILE PIGMENT OUTPUT 24 HOURS	HEMOGLOBIN INJECTION 24 HOURS	HEMOGLOBIN RECOVERY BY BLEEDING	BLOOD HEMOGLOBIN LEVEL	BLOOD VOLUME	BILE VOLUME	WEIGHT
	mgm.	*gm.*	*gm.*	*per cent*	*cc.*	*cc.*	*kgm.*
Feb. 18	46					162	
19	58		0.8	52	1,372	146	16.2
20	55					158	
21	71					170	
22	85	3	6.8	54		218	16.1
23	186	3				134	
24	175	3				182	
25	160	3				178	
26	164	3				188	
27	159	3				180	
28	147	3				178	16.2
Mar. 1	148	3	9.1	50		160	
2	172	3				156	
3	178	3	12.5	58		170	
4	156	3				214	
5	160	3				202	
6	165	3				200	
7	165	3				158	
8	161		17.8	65	1,495	166	16.1
9	79					150	
10	66		0.9	51		178	
11	62					146	
12	72					150	
13	59					160	
15			6.7	56			
17			5.2	55			
22			13.4	57	1,375		

period 28.7 grams of hemoglobin were injected and 26 grams were recovered by bleeding, a recovery of 90 per cent. At the same time 1131 mgm. of bile pigment were excreted in excess whereas 1006 mgm. were expected. The excess of 125 mgm. can undoubtedly be attributed to the disintegration of red cells in the hematoma.

Reference should be made to a single animal which has been reported previously (6) where the results differed from these recorded in this paper and others reported at that time. This particular dog (26-45), following the injection of hemoglobin, excreted only from 50 to 80 per cent of the expected amount of bile pigment. The explanation for this lower output was obvious when an autopsy was subsequently performed. A small fistulous tract had formed between the cut ends of the common bile duct

TABLE 4

Bile pigment and hemoglobin recovery after hemoglobin injection

Dog 36-108—Bag fistula, anemic

DATE	BILE PIGMENT OUTPUT 24 HOURS	HEMOGLOBIN INJECTION 24 HOURS	HEMOGLOBIN RECOVERY BY BLEEDING	BLOOD HEMOGLOBIN LEVEL	BLOOD VOLUME	BILE VOLUME	WEIGHT
	mgm.	*gm.*	*gm.*	*per cent*	*cc.*	*cc.*	*kgm.*
Apr. 8	58					180	16.5
9	65		0.9	51		158	
10	68					176	
11	64					216	
12	64	2	1.6	49	1,234	190	15.8
13	156	2				176	
14	143	2				162	
15	166	2				174	
16	160	2				176	
17	163	2				226	
18	170	2				202	
19	136	2	2.2	65	1,398	214	16.5
20	164	2	18.1			216	
21	138	2				190	
22	157	2	0.8	56		194	
23	137	2	6.4			164	
24*	137	2				200	
25	140	2.8				200	
26	200		10.4	59		210	
27	125					202	
28	104					174	
29	103		1.7	51	1,303	202	16.4
30	84					164	

* Small hematoma in leg.

and consequently variable amounts of bile had escaped into the duodenum. In spite of all precautions to ligate and remove a portion of the common bile duct at operation this complication of re-establishment of the duct by a fistulous tract occasionally occurs. Autopsies on all of the other dogs reported in this and the previous paper revealed no such communication with the duodenum, so we can be confident that we were receiving all of the daily excretion of bile.

DISCUSSION. The summary table shows clearly the uniform results obtained. It is apparent that the introduced hemoglobin is conserved by the anemic dog and that there is 90 to 100 per cent utilization to form new hemoglobin in red cells. At the same time an excess of bile pigment equivalent to the amount of injected hemoglobin is being excreted. In order to explain such a reaction, it is necessary to postulate that the pigment radicle or pyrrol aggregate is split off from the injected hemoglobin and utilized to form bile pigment in the ratio of 35 mgm. of bile pigment to 1 gram of hemoglobin. The iron and globin fractions are retained within the body in a form that should be readily used by the dog in elaborating the new hemoglobin. However, to form the new hemoglobin *new pigment radicle or pyrrol aggregate* must be combined with the conserved disintegration products. Therefore it would appear that under these conditions the body can produce the pyrrol aggregate readily.

TABLE 5

SUMMARY

Total hemoglobin injection and total recovery of bile pigment and hemoglobin in anemia

DOG		TOTAL HEMOGLOBIN			TOTAL BILE PIGMENT ABOVE CONTROL LEVEL	BILE PIGMENT EXCESS ABOVE CONTROL	
Number	Weight	Intake intravenous	Output bleeding	Output intake ratio		Equivalent to hemoglobin	Expressed as per cent of hemoglobin injection
	kgm.	*gm.*	*gm.*	*per cent*	*mgm.*		
35-15	14	42	38	91	1,295	37	88
37-75	11.5	31	27	88	965	27.5	88
36-108	16.2	42	41	98	1,414	40.4	96
36-108	16.5	28.8	26	90	1,131	32	111

Other experiments previously reported substantiate this hypothesis. When the bile fistula is combined with splenectomy (10, 11) the dogs frequently become infected with "Bartonella Canis" and they show recurrent periods of marked anemia with excess bile pigment production. A similar picture is seen when anemia is produced by means of acetyl phenylhydrazine (11). These dogs on the salmon bread diet would normally produce from 2 to 5 grams of new hemoglobin per week. Instead of this low production we find them producing as much as 40 or 50 grams of hemoglobin a week, which is an amount comparable to an anemic dog being fed with liver. The dogs apparently retain the iron and globin resulting from the destruction of red cells and promptly utilize it to form these large amounts of new hemoglobin. The necessary pigment radicle or pyrrol aggregate never appears to be lacking. The question arises immediately as to the source of this pigment radicle. It seems unlikely that it comes

from body stores as under the stress of continued anemia due to bleeding such stores would eventually become depleted. We are in favor of the idea that the body can readily synthesize this pigment complex from amino acids derived from the diet.

This ability of the body to synthesize the pigment radicle makes it apparent that the limiting factors in the formation of hemoglobin are not so much the pigment portion but rather the iron and protein stores of the body. Experiments in this laboratory (15) have shown that globin derived from dog, horse, and sheep hemoglobin is effectively utilized when injected intravenously or fed with the formation of new hemoglobin. That limitation of iron results in inability of the body to form new hemoglobin has been demonstrated (3). Recent data (4) show also that when the body has adequate iron supplies but inadequate protein stores there results an impairment in hemoglobin formation.

That bile pigments come from the disintegration of hemoglobin from red cells is firmly established. But this question must be answered. Does

TABLE 6

Actual and estimated bile pigment output

BILE PIGMENTS	DOG 35-15	DOG 37-75	DOG 36-108
	mgm.	*mgm.*	*mgm.*
Actual	72	67	74
Estimated	56	48	51
Difference	16	19	23

all the bile pigment that is excreted daily come from this source alone? *Muscle hemoglobin* must also be considered as a possible source as it must suffer wear and tear, just as is true for red cells. It is known (21) that parenterally introduced muscle hemoglobin is converted into bile pigment. Data relating to the length of life of the red cells in dogs (8) enable us to at least theorize as to how much of the daily excreted bile pigment might come from red cell or muscle hemoglobin. In the bile fistula dog the red cells exist in the blood stream for an average of 120 days which means that each day about 0.8 of 1 per cent of the circulating red cells are destroyed. Using this as a basis we can estimate how much bile pigment coming from destroyed red cell hemoglobin should be eliminated by a normal dog. The amount actually excreted can be determined during control periods. We have made this comparison in these dogs and the actual and estimated amounts are given in table 6.

It is within reason to suspect that the amount of bile pigment 16 to 23 mgm. in excess of the estimated amount is from muscle hemoglobin. On

the basis of the above figures, it would appear that under normal conditions from one-third to one-fourth of the daily excreted bile pigment comes from muscle pigment and the remainder from red cell hemoglobin. Further studies must be continued in order to determine, if possible, just how much of the daily excreted bile pigment does come from muscle hemoglobin.

SUMMARY

A severe experimental anemia due to bleeding may be combined with a bile fistula and enable one to follow simultaneously new hemoglobin production and bile pigment output under carefully controlled conditions. Dogs can be kept in perfect physical condition with normal activity and uniform weight under these conditions, provided bile and a suitable diet are furnished.

As the result of the anemia more young red cells are in the circulation and consequently fewer red cells are disintegrating and the daily bile pigment output is reduced.

Upon the injection of laked hemoglobin the dog utilizes it to form new hemoglobin in an equivalent amount (90 to 100 per cent) and concurrently there is a return (90 to 100 per cent) of the injected hemoglobin in the form of bile pigment.

To explain this reaction we suggest that from the injected hemoglobin is split off the pyrrol aggregate to form bilirubin and from the iron and globin fractions plus newly formed pyrrol aggregates there is formed new hemoglobin which appears in the circulating red cells. It appears that the body can synthesize the pyrrol aggregate in considerable amounts in an emergency due to anemia.

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PLASMA POTASSIUM CONTENT OF CARDIAC BLOOD AT DEATH[1]

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Potassium changes the properties of the surface layers of plant cells when its concentration passes a certain point (Osterhout, 3). The effects of lower concentration of potassium chloride on Nitella are perfectly reversible even after several hours of contact. With 0.05 M potassium, however, this reversibility ceases (Osterhout, 3).

The animal cell seems to be more sensitive to potassium as judged by the effects of lower concentrations on the electrocardiograph tracings in the dog (10), the cat (2), and the human (7). In the dog cardiac arrest occurs at a concentration between 0.014 and 0.016 M. It has been suggested that this is the critical concentration of potassium at which the heart stops, the evidence being obtained from intravenous injections of potassium salts into healthy dogs.

In pathological states caused by intestinal obstruction, intestinal fistulae, hemorrhage, and various types of trauma (table 1), the concentration of potassium in cardiac blood at the time of death varied between 0.0095 and 0.0114 M. in the cat (5, 6, 11, 12). In four dogs poisoned with potassium this average concentration was a little higher, 0.0152 M (4).

Winkler, Hoff and Smith (10), on the basis of their work on dogs, in which cardiac arrest was shown to be associated with a concentration of potassium between 14–16 mM. per liter suggested that there is a wide margin of safety for the human being, "since serum potassium would have to be increased by some 9 mM. per liter to reach a fatal level."

No such potassium concentration in the cardiac blood of humans has been found in a series of cases studied during the past eighteen months.

METHOD. At the time of death, heart's blood was withdrawn by cardiac puncture into a sterile dry syringe. From 5 to 6 cc. of this sample were introduced into a Sanford-Magath hematocrit tube containing heparin (Connaught); gently mixed, capped, and centrifuged at 2,000 r.p.m. for one hour. The plasma was removed *immediately* from the cells, and the

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potassium content of 0.5 ml was determined by a modification of the argenticobaltinitrite method (8, 9), the final color being read on the Evelyn Photoelectric Colorimeter with the appropriate filter.

With this method 17.2 mgm. per cent (0.0044 M) represents our normal value derived from determinations done on sixty healthy voluntary donors (table 2).

TABLE 1

Cardiac blood potassium

NUMBER OF ANIMALS	LESION	RANGE	AVERAGE	AVERAGE	AVERAGE M
		Cats			
		mgm. per cent	*mgm. per cent*	*mM./l.*	
4	Intestinal obstruction	33.8–66.6	44.5	11.4	0.0114
2	Intestinal fistula	40.6–45.5	43.1	11.0	0.0110
4	Hemorrhage	25.0–57.4	46.5	11.9	0.0119
4	Trauma	30.6–41.0	37.2	9.5	0.0095
14			42.8	10.9	0.0109
		Dogs			
4	Poisoned with intravenous isotonic KCl	26.0–99.5	59.5	15.2	0.0152

TABLE 2

*Plasma potassium of normal venous blood**

Sixty donors

Average (mean)	17.2 mgm. per cent
Median	17.2 mgm. per cent
Range	13.5–21.5 mgm. per cent
Standard deviation	0.33 mgm.
Coefficient of variation	1.9 per cent

* This group comprised 50 males and 10 females. Each value represents the mean of two aliquots of the original sample, 0.5 ml. of plasma being used.

Discussion. Cardiac arrest appears to be associated with different concentrations of potassium, not only for different species but also for different individuals within the species. Plant cells seem to withstand higher concentration of potassium than do animal cells.

The narrow range of potassium between 17.2 mgm. per cent (0.0044 M) for normal circulating venous blood and 29.8 mgm. per cent (0.0076 M) for cardiac blood plasma at death indicates possibly that human cardiac

muscle is more susceptible to variations in concentration than certain plant and animal cells.

TABLE 3

Potassium content of cardiac blood at death

NUMBER	DATE	INITIALS	AGE	SEX	HOSPITAL NUMBER	DIAGNOSIS	OPERATION	PLASMA K
								mgm. per cent
1	8/29/37	W. N.	62	M	527186	Multiple fractures and contusions; laceration of aorta	Debridement	28.5
2	9/23/37	V. M.	9	M	530304	Multiple fractures. Hemoperitoneum	None	29.5
3	10/14/37	N. L.	28	F	530208	Idiopathic gastro-intestinal hemorrhage	Exploratory	24.0
4	11/23/37	F. F.	58	M	536668	Perforated duodenal ulcer	None	31.9
5	2/24/38	D. P.	25	F	542008	Paraganglioma of adrenal cortical tissue	Partial resection. Operative death	34.1
6	6/ 5/38	M. L.	45	F	374569	Chronic cholecystitis, cholelithiasis, subphrenic and subhepatic abscesses	Cholecystectomy, incision and drainage abscesses	28.6*
7	9/12/38	J. Mc.	70	M	556217	Carcinoma of colon with metastases to liver	Exploratory	26.3
8	9/27/38	M. K.	26	F	550662	Intestinal obstruction complicating pregnancy	Ileostomy	28.9
9	11/ 4/38	L. W.	31	F	549037	Mesenteric thrombosis	Enterectomy	38.0
10	11/ 7/38	E. Mc.	62	F	564918	Acute pancreatitis	None	31.6
11	12/13/38	L. V.	73	F	560067	Diabetic gangrene	Amputation	32.6
12	2/17/39	L. H.	58	F	566522	Carcinoma of breast	Mastectomy	26.1
13	2/24/39	A. M.	55	M	572368	Pneumonia, type III		27.9
Average								29.8

* Not separated from cells immediately.

Standard deviation from the mean 3.6 mgm. Coefficient of variation 14.5 per cent.

SUMMARY

1. In cats dying from varied types of induced shock, the average concentration of potassium in the heart's blood taken at the time of cardiac arrest was 42.8 mgm. per cent (0.0109 M).

2. In dogs following intravenous injections of isotonic potassium in lethal doses, the concentration amounted to 59.5 mgm. per cent (0.0152 M) (table 1).

3. The average venous plasma potassium in sixty young human adults was 17.2 mgm. per cent (0.0044 M) (table 2).

4. The average plasma potassium of cardiac blood taken at death was 29.8 mgm. per cent (0.0076 M) (table 3).

We wish to express our appreciation to both the attending and resident staff of the hospital for their coöperation in securing the cardiac blood samples.

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SODIUM CHLORIDE AND DIABETES INSIPIDUS

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That some defect in the metabolism of NaCl is involved in the etiology of diabetes insipidus (d.i.) has long been suspected. Meyer (1905, 1912) suggested that the disease was due to a lack of ability of the kidneys to excrete a urine concentrated in NaCl. And it has long been known that if an excess of NaCl is administered to humans with d.i., there follows an increase in the severity of the disease (reviewed in Fitz, 1914; Rosenbloom and Price, 1916; Oehme and Oehme, 1918); conversely, it is common clinical practice to reduce the salt intake of individuals with d.i. in order to reduce the magnitude of the fluid exchange.

Experimentally also, it has often been shown that an operative d.i. is greatly exaggerated by NaCl administration (Bailey and Bremer, 1921; Curtis, 1924; Fisher, Ingram and Ranson, 1938; Bellows, 1938; Swann and Penner, 1939). Giving d.i. animals salt water to drink results at times in enormous fluid exchanges: Bellows (1938) has reported such a dog to drink 1000 cc. of saline per kilogram in one day; the author (Swann and Penner, 1939) has reported one rat to drink 1700 cc. per kilo per day, and in another study (Swann, 1939a) one rat was discussed which drank 2397 cc. per kilo in one day.

In seeking to account for this effect and for an elucidation of the rôle of NaCl in the etiology of d.i., an observation by Curtis (1924) furnished a suggestion. He found that experimental d.i. in the dog was much improved if the animal was fasted. This has been denied (Bailey and Bremer, 1921) and confirmed (Fisher, Ingram and Ranson, 1938). Curtis also reported that the severity of his dogs' d.i. was roughly proportional to the amount of NaCl ingested in the animals' food. In the d.i. rat, Richter has shown (1938) that the fluid exchange parallels the quantity of food ingested. As previously suggested by the author (Swann and Penner, 1939), it seemed possible that the reduction of the d.i. by fasting might be primarily due to the reduction of the NaCl intake which fasting causes. This hypothesis was tested by controlling the amount of NaCl ingested during induced d.i. in the rat.

The technics used have been previously detailed (Swann and Penner, 1939). D.i. was produced by removing the posterior hypophysis (post-

hypophysectomy). Each animal was kept in an individual metabolism cage. The fluid exchange was measured daily. In this paper only the intakes of fluid will be reported, but the output corresponds in all cases to the intake. Other technical details will be mentioned below.

RESULTS. *Fasting experiments.* It was first determined whether fasting ameliorated d.i. in the rat, as it does in the cat and the dog. A group of 9 rats, posthypophysectomized 4 weeks previously, were fasted for two days. During the week before the fast, the average intake per rat per day was 26 cc.—an intake about 3 times normal. During the fast, this dropped to 13 cc. per rat per day for the first day and the same for the second. Evidently fasting ameliorates the d.i. of rats as it does other species.

It was also found that the ameliorating effects of fasting on d.i. could be completely prevented by giving NaCl. A group of 4 d.i. rats, which had averaged an intake of 33 cc. of water per rat per day for the previous week, were fasted for 24 hours. During the fast they were given 240 mgm. of NaCl by mouth, divided into two doses, in concentrated solution. This quantity of NaCl is about the amount ingested daily by the rat (see below). This treatment resulted in an ingestion of 30 cc. of water per rat. The NaCl obviously prevented the ameliorating effects of the fast, for the d.i. continued unchecked.

Experiments with controlled NaCl intake. From the above experiment, it was concluded that the ameliorating effect of fasting on d.i. could be ascribed to the fact that the animal ingests no NaCl during the fast. In order to clinch the matter, the converse experiment was done, i.e., giving the animals food which contained minimal amounts of NaCl to see if this prevented the d.i. The NaCl low diet of Orent-Keiles, Robinson and McCollum (1937) was used with slight modifications. Their diet contains cane sugar, butter fat, washed casein, a special salt mixture, viosterol and a specially prepared vitamin B mixture. They reported it to contain 0.002 per cent sodium. The diet here used was the same, except that for the prepared vitamin B was substituted flavin-phosphate; it was found to contain 0.008 per cent NaCl. To make this diet relatively normal in composition, 5 per cent dried yeast was added and 1.5 per cent NaCl. These two diets will be designated, respectively, the "low NaCl diet" and the "normal NaCl diet."

Six rats were given the normal NaCl diet for 3 days and then posthypophysectomized. A week later, their diet was changed to the low NaCl diet—i.e., all food elements were essentially the same except that the NaCl intake was abruptly reduced from about 250 mgm. per rat per day to 1.3 mgm. per rat per day. The effect of these manoeuvers on the water intake per rat per half-day is shown in figure 1. Measurements were made every 12 hours in order to bring out more clearly the "transient phase" of d.i. in the rat (see below). Figure 1 also shows the average

amounts of NaCl and of food ingested per rat per half-day. The graph shows that when on day 10 (half-day 19) the NaCl intake was lowered, the fluid intake abruptly dropped nearly to the preoperative normal, even though the amount of food ingested remained constant.

Figure 1 also shows that the three phases of experimental d.i. can be observed in the rat. These consist of an early postoperative rise in the fluid exchange, then a drop to normal, and finally a rise again to high levels. The three phases have been called (Fisher, Ingram and Ranson, 1938), respectively, the transient phase, the interphase, and the permanent phase. In the cat or dog they are more prolonged than in the rat, the first two lasting roughly 6 days. In the rat, as figure 1 shows, they last about 24 hours.

Now the experiments described above were all done when the rats were in the permanent phase of d.i. They showed that the continuation

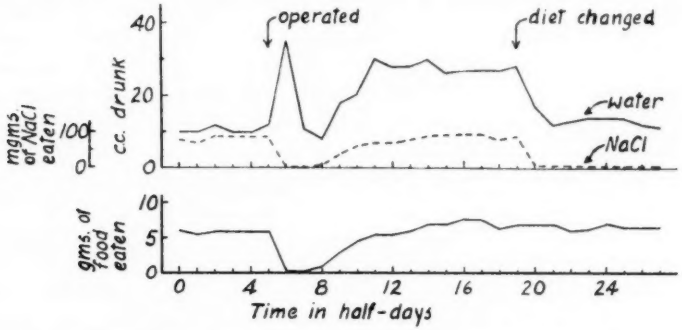

Fig. 1. The effect of reducing the NaCl intake on diabetes insipidus

of this phase is dependent upon the ingestion of NaCl. Figure 1 shows, however, that the transient phase is not dependent on the NaCl ingested; it occurs when the animal is not eating. However, the onset of the permanent phase corresponds in figure 1 with the onset of appetite and the ingestion of food and NaCl. This suggested that the onset, as well as the continuation, of the permanent phase is dependent on the ingestion of NaCl. This was put to more rigid test: one group of 6 rats were given the low NaCl diet from the start; another group of 6 rats were fasted for 7 days following the operation. Unoperated control groups of 6 rats were also observed. The average intakes per rat per half-day of these animals are shown in figures 2A and 2B. The posthypophysectomy was done at *O*. Figure 2A indicates that even though the NaCl intake was much reduced (to about 1/200th the normal intake), a degree of d.i. still was observable in the permanent phase, i.e., the fluid intake of the operated group exceeded that of the normal group about 3 times.

It seemed possible that even the small amount of NaCl in the low NaCl diet was responsible for this slight residual d.i. In order to reduce the NaCl intake to zero, 6 animals were operated and then fasted for 7 days. Their water intakes from operation and those of an unoperated control group are shown in figure 2B. Even with the treatment, a degree of d.i. still occurred, the animals drinking water from the fourth to the eighth half-day at about 3 times the rate of the controls. In summary, mild d.i. occurred even when no NaCl was ingested, but the very large fluid exchanges commonly seen in d.i. were dependent on the ingestion of NaCl.

Certain other experiments have been done, however, which do not agree with this conclusion any too well. When operated rats which had been given either no NaCl or small amounts of NaCl in the diet were given the normal NaCl diet, the d.i. produced was a relatively mild one. Thus, the rats of all three groups discussed in figures 1, 2A, and 2B were given, at the termination of the experiments discussed and illustrated

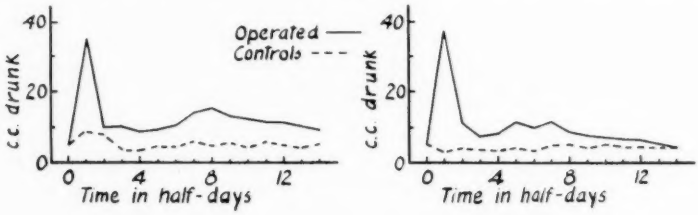

Fig. 2A Fig. 2B

Fig. 2A. The effect of a low NaCl intake on diabetes insipidus
Fig. 2B. The effect of fasting on diabetes insipidus

above, the normal NaCl diet. The intake of the group of figure 1 (eating the low NaCl diet) rose from about 12 cc. per rat per half-day to 18 cc. The intake of the group of figure 2A (also eating the low NaCl diet) rose from 9 cc. per rat per half-day to 21 cc. The intake of the group of figure 2B (fasted) rose from 4 cc. per rat per half-day to 17 cc. It was expected that this treatment would restore the fluid intake to the d.i. level of figure 1, i.e., about 30 cc. per half-day, but this did not occur in any group's average, although it did in a few individual cases. An attempt will be made to account for this later in this paper.

Discussion. In order to explain why a low food intake ameliorates d.i., Richter (1938) postulated that the general metabolism must be maintained to get the maximal fluid exchange. It has here been demonstrated that the NaCl ingested is the critical variable in fasting and not some "general metabolic level." Since only very light d.i. is obtained when no NaCl is ingested, it seems clear that in d.i. there is some defect in NaCl metabolism and that the defective water metabolism is dependent

on and secondary to this defective NaCl metabolism. This explains why NaCl aggravates d.i. so extremely: the added NaCl aggravates an already defective NaCl metabolism.

It is clear, however, that ingested NaCl is not responsible for the transient phase of d.i. To account for this difference there are several possibilities. Meyer's old theory (1905, 1912) that the d.i. animal cannot excrete a urine concentrated in NaCl has been disproved (Socin, 1913; Bailey and Bremer, 1921; Curtis, 1924; Swann, 1939a), but has in certain aspects some merit (Swann, 1939a). Another hypothesis that appears favorable at this writing and that is now being tested is that in d.i. the threshold of excretion of NaCl drops to a lower level than normal. This theory would explain why the transient phase occurs independently of NaCl ingestion, for the animal loses in the transient phase its own NaCl through its own kidneys. When a lower level is reached, the diuresis diminishes (the interphase). If the animal is not fed, a degree of diuresis persists because NaCl is being drawn into the blood stream from endogenous sources. When the appetite returns and with it the ingestion of food and NaCl, the permanent phase starts and persists as long as salt is ingested. This hypothesis accounts for all three phases of d.i.; it accounts for the NaCl appetite that can be demonstrated in d.i. (Swann, 1939b); and it accounts for the effects of nephrectomy (Richter, 1934; Swann, 1939a) in all but one aspect of d.i.

Such a theory assumes that in d.i. the polyuria is primary. There is much support for this theory (reviewed in Fisher, Ingram and Ranson, 1938), but some strong dissent (reviewed in Bellows and Van Wagenen, 1938). Also, in opposition to the theory are reports (Curtis, 1924; Peters, 1935) of a slight hyperchloremia and hypernatremia in d.i. In another paper (Swann, 1939a), also, it has been shown that in certain aspects of d.i. the polyuria is clearly primary but in other aspects the polydipsia is clearly primary. It is felt that a closer investigation of the metabolism of NaCl in the condition will help resolve many of these paradoxes.

The experiments which did not fit the proposition that NaCl is the important variant in the condition were mentioned. They indicated that NaCl administration after a period of NaCl privation caused only a mild d.i. in the posthypophysectomized rat. It is possible that a low rate of NaCl ingestion may affect the condition permanently. Another possible explanation is to be found in the behavior of the rat after this operation. The d.i. thus induced is not permanent but lasts only about 8 weeks (Dodds, Noble and Williams, 1937; Swann, 1938). This improvement we have ascribed to secretion of the anti-diuretic hormone by the stalk, etc. Such a condition makes the rat rather unsatisfactory for this type of work, since time itself will improve the condition. It is this factor which we think responsible for the relative ineffectiveness of salt given

after the period of salt deprivation. The changes in rate of NaCl ingestion were made 2 or 3 weeks postoperatively. They returned the animal's fluid intake to about 40 cc. per day, and this amount is about the expected intake for this time postoperatively (Swann, 1938). The matter could be more clearly settled by technics causing a truly permanent d.i., i.e., denervation of the pars nervosa.

SUMMARY

In the permanent, but not the transient, phase of diabetes insipidus, the onset and continuance of the large fluid exchange are to a large extent dependent upon the ingestion of NaCl. If none is ingested, as in fasting or with diets low in NaCl, the diabetes insipidus is almost, but not entirely, prevented.

It is suggested that the changes in water metabolism in diabetes insipidus are secondary to changes in NaCl metabolism.

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RENAL AND VASCULAR RESPONSES TO EPINEPHRINE INJECTIONS IN GLOMERULAR AND AGLOMERULAR FISH[1]

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Infusions of epinephrine in anesthetized dogs result in either an oliguria or a polyuria depending on the rate at which the infusions are made (1). A possible explanation for the dual effect of epinephrine can be advanced from the work of Richards and Plant (2) and Winton (3), who suggested that with a low concentration of epinephrine in the blood stream the efferent arterioles are constricted and induce polyuria; but with a higher blood concentration after large injections the afferent, or both the afferent and efferent, arterioles are constricted, producing oliguria. In order to determine the rôle of the glomerulus in the urinary response to epinephrine the present investigation was undertaken.

METHODS. The puffer (Spheriodes maculatus) possessing a glomerular kidney, and the toadfish (Opsanus tau) possessing an aglomerular kidney (4), were used in the experiments. The animal was anesthetized by placing it in a urethane solution in sea water (1 per cent for the puffer; 2 per cent for the toadfish) and then was transferred to a more dilute urethane solution (0.5 per cent for the puffer; 1.0 per cent for the toadfish). It was pinned on its back with its gills under water and with oxygen bubbling through the water, or directly through the mouth and over the gills. The temperature of the water was constant within 2.0°C. during any one experiment. One ureter was cannulated and the urine flow determined by frequent observations of the meniscus in the cannula; the urine rate was later plotted as cu. mm. per 100 grams body weight per hour (cu. mm./100 grams BW/hr.). The blood pressure in the coeliac or subclavian artery was determined in several experiments with a mercury manometer, 10 per cent sodium citrate being used as the anticoagulant in the manometer. The heart rate was counted in the majority of the experiments. All of the injections were made through a cannulated gonadal vein.

[1] A preliminary report of this work appeared in the Proceedings of the Society for Experimental Biology and Medicine, **38**: 299, 1938.

[2] Contribution no. 208.

After determining a normal urine rate, dilute sea water equivalent in volume to that of the epinephrine solution to be used subsequently was injected as the control. The volume never exceeded one cubic centimeter. When the effects of the control solution had worn off the epinephrine solution (Parke-Davis Adrenaline Chloride) varying in concentration from 1:1000 to 1:50,000 in dilute sea water, was injected and the response of the urine rate, blood pressure and heart rate observed and recorded.

RESULTS. The results of one of the experiments on the puffer are given in figure 1. At *B* 0.5 cc. of the control solution was injected. No significant change occurred in the urine rate or the blood pressure. The heart

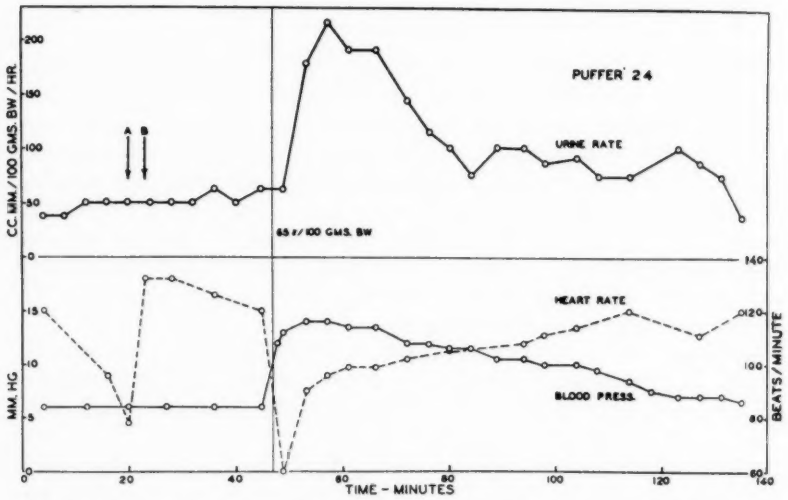

Fig. 1. Urine, arterial blood pressure, and heart rate responses of the puffer to control and epinephrine injections.

rate as a rule did not vary greatly; but in this experiment the decrease in heart rate just before the injection of the control solution was evidently the result of asphyxia because after the oxygen flow was increased at *A* the heart recovered. This response of the asphyxiated heart to oxygen was observed in a number of animals.

In contrast to the control solution the urine rate after epinephrine increased significantly, an increase of 340 per cent occurring in puffer 24. The blood pressure also increased even though the heart rate fell from 120 to 60 beats per minute. This fall in heart rate usually occurred after every epinephrine injection.

A summary of the urine and cardio-vascular responses in the experi-

TABLE 1

Urine and cardio-vascular responses to epinephrine and control injections in the puffer (Speriodes maculatus)

ANIMAL NUMBER	WEIGHT	DOSAGE	PREVIOUS URINE RATE	URINE RESPONSE TO:		CARDIAC RESPONSE IN BEATS PER MINUTE* TO:					
				Epineph.	Control	Epineph.			Control		
						1	2	3	1	2	3
	grams	*γ/100 grams body wt.*	*cu. mm./ 100 grams BW/hour*	*per cent†*	*per cent†*						
20	165	242	122	280	100	117	100	114			
19	156	160	3	11,600	100	36	60	64	50	50	
18	247	105	3	9,500	100	44	36	76	52	40	
14	134	75	35	930	170	42	36	42	62	46	22
17	144	70	6	535	230	62	32	60	64	70	60
24	153	65	51	390	100	124	60	120			
3	160	63	26	1,960	250						
1	177	57	21	525	260						
21	217	46	50	215	150	100	48	90	90	74	84
16	250	40	3	2,360	400	96	62	86	84	88	100
9b	152	32		1,880		84	48	68			
15	198	25	55	330	240	86	66	90	90	90	76
6	241	21	26	690	190	90	42	88			
9a	152	16	5	980	100	54	42	80			
8	228	11	11	3,860	100	64	62	70	50	28	42
7	237	10	5	690	820	86	76	84	22	38	84
12a	163	6	31	265	110	82	56	84	84	82	80
10	195	5	4	3,275		76	24	72			
12c		3		490		72	66	76			
23	203	0	39								

			BLOOD PRESSURE RESPONSE IN MM. Hg* TO:										
			Epineph.			Control							
			1	2	3	1	2	3					
24	153	65	6	14	7	6	6	6					
21	217	46	9	18	11	11	13	7					
22a	219	23	16	29	13	13	13	13	82	78	96	100	102	94
22b		23	13	17	15	16	17	16	88	78	94			
22c		23	15	25	12				100	70	96			

Where several injections were made in one animal they are indicated by a letter after the number identifying the animal.

* In columns 1 are listed the arterial blood pressures and heart rates just preceding the injection; in columns 2 the greatest changes that occurred after the injection; in columns 3 the readings after recovery.

† Per cent of urine rate just preceding injection.

ments carried out on the puffer is given in table 1. In every case the urinary response to epinephrine is much greater than the response to an equal volume of the control solution. In some cases a 100-fold increase in the urine rate occurred, these tremendous increases occurring in those experiments in which the urine rates before the injection were low. At no time did a pronounced oliguria occur, even with the larger doses of epinephrine, as it did in dogs (1). In a few of the experiments not listed no urine flow was evident for 30 minutes before, or after, the injection of the control solution; but after the epinephrine injection a polyuria resulted.

The urine and cardio-vascular responses of the toadfish are given in figure 2. Simultaneous determinations of urine rate, blood pressure, and heart rate were not carried out on the same animal; therefore, the experi-

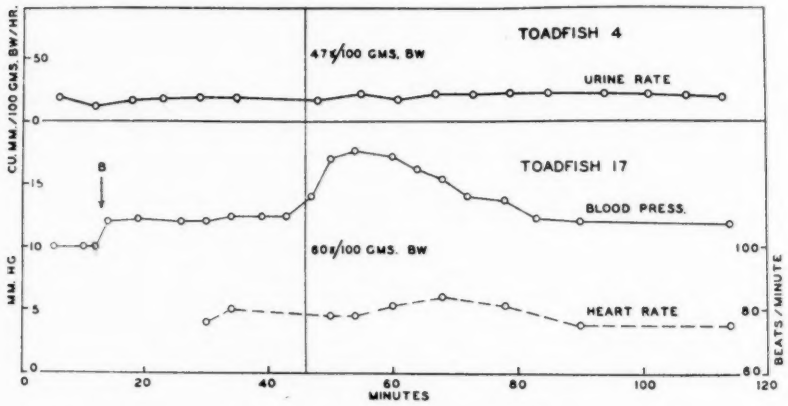

Fig. 2. Urine, arterial blood pressure, and heart rate responses of the toadfish to control and epinephrine injections.

ments performed on two animals weighing the same and receiving approximately the same amount of epinephrine are given in this figure. The effects of the control injection on urine rate in toadfish 4 are not shown but the increase was from 22 cu. mm. to 25 cu. mm./100 grams BW/hour. The urinary response to the epinephrine solution in this animal was even smaller. The blood pressure response of toadfish 17 to the control solution (injected at *B*) and to the epinephrine, and the heart rate response to the epinephrine are also shown. The heart rate usually increased after both solutions, the increase being slightly greater after the epinephrine. This response is in contrast to that of the puffer.

Table 2 summarizes the urine and cardio-vascular responses of the toadfish to the control and epinephrine solutions. There is no great difference in the urine response to the two solutions, a point differing significantly

from the response of the glomerular kidney of the puffer. Unlike Bieter's (5) results, at no time did the toadfish show a prolonged and significant polyuria after the epinephrine injections. Bieter reported that magnesium chloride, magnesium sulfate, sodium sulfate, and urea injected intraven-

TABLE 2

Urine and cardio-vascular responses to epinephrine and control injections in the toadfish (Opsanus tau)

ANIMAL NUMBER	WEIGHT	DOSAGE	PREVIOUS URINE RATE	URINE RESPONSE TO:		CARDIAC RESPONSE IN BEATS PER MINUTE† TO:					
				Epineph.	Control	Epineph.			Control		
						1	2	3	1	2	3
	grams	*γ/100 grams body wt.*	*cu. mm./ 100 grams BW/hour*	*per cent**	*per cent**						
11	298	67	6	150	165	24	50	30	26	32	30
12a	328	61	10	130	170	58	60	50	74	64	72
6	343	58	3	125	260	42	44	34	52	48	54
7	360	56	3	135		48	54	50			
10	368	55	9	65	100	54	60	66	56	60	56
5	406	49	2	100		34	40	38			
13	420	48	3	270	135	60	66	60	56	58	50
4	423	47	21	110	115	56	60	60	60	60	60
8a	504	40	4	125	100	58	66	58	62	66	58
12b	328	15		80		46	36	40			
9	351	14	13	80	125	30	28	28	60	46	56
8b	504	10		80		44	54	36			
14	402	5	11	120	165	56	60	56	50	56	52
15	415	0	25								

ANIMAL NUMBER	BLOOD PRESSURE RESPONSE IN MM. Hg† TO:						CARDIAC RESPONSE IN BEATS PER MINUTE† TO:					
	Epineph.			Control			Epineph.			Control		
	1	2	3	1	2	3	1	2	3	1	2	3
17	12	18	11	10	12	12	80	78	76			
16	12	16	13	9	12	12	68	78	84	68	66	68

Where several injections were made in one animal they are indicated by a letter after the number identifying the animal.

* Per cent of urine rate just preceding injection.

† In columns 1 are listed the arterial blood pressures and heart rates just preceding the injection; in columns 2 the greatest changes that occurred after the injection; in columns 3 the readings after recovery.

ously induced a polyuria, and attributed this response to the increased secretion of these substances by the tubules. Evidently the mode of action of the salts in Beiter's work and the epinephrine in the present work is different.

The urine rates of the anesthetized puffer and toadfish here reported are similar to those reported by Grafflin (6) for the sculpin and toadfish. Calculated as cubic centimeter per kilogram body weight per day he reported the urine rate for the sculpin to be usually less than 4 cc., and that for the toadfish to be below 2.5 cc. From a review of the literature Grafflin gave the rates for other marine teleosts as follows: wrymouth, 1.5 to 11.0 cc., sculpin, 3.2 to 57.0 cc.; goosefish, 12.9 to 54.4 cc.; toadfish, 0.6 to 9.4 cc. According to Grafflin the higher rates are the result of possible injury to the fish due to handling.

COMMENT. Although epinephrine injections induced a rise in the arterial blood pressure in both species of fish used in the experiments, the urinary response differed in that the glomerular kidney showed a polyuria while the aglomerular kidney showed no significant change from that of the control injection. This difference in the urinary response may be correlated with the presence or absence of the glomerulus and its arterioles. With the afferent and efferent arterioles present the renal mechanism which responds specifically to epinephrine gives one of the responses, namely, a polyuria, that the dog's kidney gave (1). Without the glomerulus and its arterioles no significant changes occur after epinephrine injections. Unlike that observed in the dog (1) a persistent oliguria was not observed in the experiments on the glomerular kidney, even with very large doses of the drug.

The cardiac response of the puffer to epinephrine is similar to the findings of Lutz (7), who reported that epinephrine in concentrations of 1:50,000 to 1:25,000 in the immersion fluid produced a slowing or temporary complete inhibition of the beat of the isolated sinus-auricle preparation of Elasmobranch fish. He explains the inhibition as the response of an unbalanced parasympathetic mechanism in a heart which lacks a sympathetic accelerator innervation. This explanation may hold for the puffer but the toadfish seldom exhibited a cardiac slowing.

It is not inferred that marine teleosts and mammals, or even species in the same order of teleosts, respond alike to epinephrine. Nevertheless, the data obtained in the present series of experiments suggest that the polyuria of epinephrine may be of wholly circulatory origin, and not dependent on any activity of the renal epithelium.

SUMMARY

Intravenous epinephrine injections varying in doses from 242 to 3 micrograms per 100 grams body weight in the puffer (Spheriodes maculatus) possessing glomerular kidneys resulted in a polyuria; similar injections in the toadfish (Opsanus tau) possessing aglomerular kidneys, in which the dose varied from 67 to 5 micrograms per 100 grams body weight resulted in no significant polyuria. Both species showed a rise in arterial

blood pressure, but the heart rate of the puffer decreased significantly while the heart rate of the toadfish increased slightly. The hypothesis is advanced that the glomerulus with its afferent and efferent arterioles is necessary for the epinephrine polyuria to occur.

The author wishes to express his gratitude to Dr. E. F. Adolph and Dr. H. S. Mayerson for their helpful criticisms in the preparation of this manuscript; and to Dr. Henry B. Bigelow, Director of the Woods Hole Oceanographic Institution, for having been permitted to do the experiments at the Institution.

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THE EFFECTS OF RENIN, PITRESSIN, AND PITRESSIN AND ATROPINE ON RENAL BLOOD FLOW AND CLEARANCE

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The purpose of the present study was to investigate the effects on the circulation of the kidneys of the renal pressor substance renin and of the hypophyseal pressor substance "pitressin." Renal blood flow was indirectly measured by a modification of the method of Van Slyke, Rhoads, Hiller and Alving (1934) in which phenol red (Sheenan, 1936) and inulin were used instead of urea. The specific renal effects of these substances were determined by absolute and relative changes in phenol red and inulin clearances and renal extractions.

METHODS. 1. *Transplantation of kidney and preparation of the renal vein.* Under Amytal (Iso-amyl Ethyl Barbituric Acid, Lilly) anesthesia, an incision was made parallel and 5 cm. from the spine beginning 3 cm. below the last rib. The abdominal muscles were split and the kidney delivered through the opening. The hilus and vessels were freed of fat. A pocket was made under the skin into which the kidney was then inserted. Four silk stay sutures were placed between the abdominal musculature and the tough subcutaneous tissues to fasten the kidney with its upper pole at right angles to the spine. A gutter was prepared for the renal vessels and ureter by suturing the abdominal muscles flush to the subcutaneous tissues of one edge of the wound. The muscles on the opposite side were sutured to the subcutaneous tissue about 3 cm. from the edge, leaving the skin free to cover the open trough. With the kidney in position, the renal vessels and ureter should lie free within this groove. The skin over the kidney was closed by a subcutaneous stitch which did not include the gutter. The entire wound, including the gutter, was then closed by a continuous fine silk suture. A large cotton dressing was held in place with collodion and bandage.

This operation has the advantages that it is relatively easy to perform and that, with reasonable care, almost no failures occur.

The opposite kidney was removed when the wound over the explanted kidney had completely healed. About three months were allowed for

hypertrophy of the remaining explanted kidney to near completion, and during this period, the animal was trained to lie quietly fastened to a dog-board for periods of more than one hour.

The diet consisted of daily feeding of dog-biscuit (Purina Dog Chow) and weekly supplements of lean meat. All experiments were done after 18 hours of fasting and at least 36 hours after meat had been fed.

2. *Determination of inulin.* Inulin was determined in most of our experiments by the method of Corcoran and Page (1939) but, in a few earlier experiments, as reducing substance after acid hydrolysis by the method of Shaffer and Somogyi (1933) after acid hydrolysis of plasma filtrates (Somogyi, 1930) and urine dilutions which had been treated with yeast (Smith, Goldring and Chasis, 1938).

3. *Determination of phenol red.* Plasma phenol red was determined in the Evelyn photo-electric colorimeter (Evelyn, 1936), by a method suggested to us by Dr. K. A. Evelyn. One cubic centimeter of plasma was placed in a colorimeter absorption tube, and mixed with 5 cc. of 0.1 N NaOH. The tube was allowed to stand for 20 and not more than 40 minutes to allow the complete formation of alkaline methemoglobin from whatever hemoglobin was present, while avoiding the turbidity which develops with prolonged standing. Color density (L value)[1] of the solution was found by comparison with hemoglobin-free sample of plasma taken before the infusion of phenol red. The density was first determined using color filter no. 540 (transmission 515 to 570 millimicra), at which both phenol red and alkaline methemoglobin have maximum light absorptions. The density is then found using filter no. 660 (transmission 635 to 720 millimicra). With filter no. 660 alkaline methemoglobin shows 1/3.87 times the color density it had with filter no. 540, while phenol red in the concentrations used is transparent. The color density (L value) found with filter no. 660 was therefore multiplied by 3.87 to yield the color density the alkaline methemoglobin had with filter no. 540. This product was subtracted from the density found with filter no. 540 to yield the color density of the phenol red present in the plasma sample. The amount of phenol red was then expressed as milligrams per 100 cc. of plasma by referring the color density to a calibration chart prepared from solutions of phenol red in plasma. The method is accurate to about one per cent with phenol red concentrations of from 0.2 to 1.5 mgm. per 100 cc. of plasma, even in the presence of gross hemolysis.

Phenol red excreted in urine was determined in the Evelyn photo-electric colorimeter from the color density of an alkalinized diluted sample of mixed urine and washings. Ten cubic centimeters of water dilution of urine-washings mixture were placed in colorimeter absorption tube and 2 cc.

[1] The term color density refers to the average optical density measured by the photo-electric colorimeter with light filters. It is represented by the symbol L.

of citrate buffer[2] added. The color density was found with reference to a water blank using filter no. 540. Phenol red content was found from the color density by reference to a calibration chart prepared from solutions of phenol red in water. The method is accurate to about one per cent with phenol red concentrations of from 0.02 to 0.2 mgm. per 100 cc. sample.

4. *The infusion fluid.* The infusion fluids used were designed to yield average concentrations in plasma of about 0.6 mgm. per cent phenol red and 75 mgm. per cent inulin. The "priming" fluid contained 12 per cent inulin, 0.1 per cent phenol red and 2 per cent Na_2SO_4 in 0.9 per cent NaCl. The infusion fluid proper contained 6 per cent inulin, 0.5 per cent phenol red and 2 per cent Na_2SO_4 in 0.9 per cent NaCl.

The inulin used in these experiments was obtained from the Pfanstiehl Chemical Co. Its pyrogens were removed by two filtrations of 15 per cent solutions in 0.9 per cent NaCl through Seitz E.K. serum filters. Sodium sulphate was added to the infusion fluid to insure moderate urine flows during the experiments. The urine flows observed during experimental periods are consequently of only relative significance.

5. *The experiment.* The animal was comfortably fastened to a dog-board. A control blood sample was taken from the external jugular vein through a 15 gauge needle and a size 4 (French scale) ureteral catheter passed through the needle into the vein. The catheter was then connected with a flask containing the "priming" fluid, 30 to 40 cc. of which were given at the rate of about 1 cc. per minute. Infusion fluid proper was administered at the same rate when the "priming" injection was completed and this infusion continued for about 20 minutes before starting the experiment.

Each experiment consisted of 3 or 4 control periods and 3 or 4 experimental periods each of about 20 minutes' duration. The bladder was washed with 0.9 per cent NaCl at the beginning of each period. A sample of renal vein blood was taken near the middle of the period and a sample of femoral arterial blood as soon as possible thereafter. In most periods the femoral arterial blood pressure was taken by means of a mercury

[2] The citrate buffer (pH 12) was prepared by mixing equal volumes of citrate buffer (Sørensen) pH 6.0 and 0.667 N NaOH. The purpose in using this mixture rather than 0.1 N NaOH, which gives identical readings, was to correct for the presence of hemolysis in some urine samples. At pH 6.0 phenol red gives little color with filter no. 540 as compared with the color given at pH 12.0. After the addition of 1 cc. of the citrate buffer at pH 6.0 to the sample, the color density obtained would be largely that of interfering substances; the subsequent addition of 1 cc. of 0.667 NaOH would give a color density equal to the sum of phenol red plus the interfering substances. Knowing the difference in color density between the two readings, one could thus determine phenol red in the presence of substances which absorb light at 540 millimicra and pH 6.0. Although it was not found necessary to make this calculation in any of our experiments, we have continued the use of the buffer mixture.

manometer connected with the needle through which the arterial blood had been withdrawn. At the end of the period the urine was removed from the bladder by suction and manual expression and the bladder washed out with two 25 cc. portions of 0.9 per cent NaCl which were similarly removed.

The blood samples were taken into syringes moistened with a solution of heparin in 0.9 per cent NaCl and rapidly transferred to flasks containing a few milligrams of dry heparin (Connaught Laboratories, 15 U. per mgm.). They were then centrifuged and the plasma withdrawn. The hematocrit value was found at the beginning, mid-point and end of each experiment from arterial blood samples centrifuged in Wintrobe hematocrit tubes. The skin temperature was recorded from points on one thigh, mid-abdomen and chest from which the hair had been clipped. These determinations were made by means of the Dermatherm (Taylor Instrument Co.) usually once in each period.

Renin or pitressin (Parke, Davis & Co., 20 units per cc.) was measured into a known amount of infusion fluid in a separate flask and the experimental periods preceded by the infusion of this mixture for 8–10 minutes before the bladder was washed and the first experimental period begun. The renin was prepared by Dr. O. M. Helmer by the method of Helmer and Page (1939). Atropine was given as atropine sulphate dissolved in 0.9 per cent NaCl injected rapidly from a syringe through the ureteral catheter into the external jugular vein. The catheter was reconnected to the infusion flask containing pitressin and the infusion continued.

6. *Calculation of results.* The values found for arterial plasma phenol red and inulin concentrations were plotted semilogarithmically against time in minutes. The probable values for the mid-points of each period and also for the times of collection of renal venous blood were found on this chart by interpolation. The renal clearance was calculated from the arterial plasma concentration at the midpoint of each period (B) and the concentration found in the mixture of urine and washings (U) by the UV/B in which V is the volume of mixed urine and washings divided by the duration of the period in minutes. The arterial concentration at the time of renal venous sampling (A) was used in the calculation of the renal extraction percentage (E_p) by the formula $E_p = A - V/A \times 100$, in which V is the concentration found in renal venous plasma. Renal plasma flow was then found by the formula C/E_p, where C is the plasma clearance. Whole blood renal flow was calculated by the formula $PF/Vp \times 100$, where PF is renal plasma flow, Vp the percentage of plasma in whole blood. Vp was obtained by interpolation of the hematocrit values semilogarithmically against time on the chart used in the calculation of arterial plasma concentrations. As the clearance was calculated in every case as cubic centimeters per minute per square meter of body surface, the renal blood and plasma flows are also expressed with reference to surface area.

Surface area was calculated by the formula of Cowgill and Drabkin (1927) as modified by Rhoads, Alving, Hiller and Van Slyke (1934).

7. *Arrangement of data.* The results are considered first with reference to the values of single control periods, which are grouped and correlated. For consideration of the effects of "renin" and pitressin the control and experimental periods of each experiment were averaged and the effects of experimental procedures expressed as average percentage variations from the mean control values. The values found in single experimental periods are expressed graphically with reference to mean control values (fig. 1).

RESULTS. 1. *Control periods.* Mean values were derived from 77 observations on 7 dogs. Standard deviation (σ) was calculated as $\sigma = \sqrt{\frac{\Sigma d^2}{n}}$ Standard error of the mean (SE_m) was found by the formula $SE_m = \frac{\sigma}{\sqrt{n}}$

TABLE 1

Correlation and regression coefficients of control periods

	r	b	PERCENTAGE OF DATA WITHIN	
			15 per cent of curve	Twice SE_m of curve
Phenol red:				
Flow/clearance	+0.90	+0.321	59	37
Flow/extraction	−0.78	−0.099	57	46
Inulin:				
Flow/clearance	0.97	0.187	85	56
Flow/extraction	−0.95	0.056	87	51

Correlation coefficients (r) regression coefficients (b) and approximate scatter of data about the curves calculated from the regression equations obtained from the data of control periods with reference to the relations of phenol red and inulin clearances and extraction percentages to renal plasma flow.

Mean whole renal blood flow was 460 cc. per square meter per minute, σ ±118 cc., SE_m ±13 cc. This renal blood flow is equivalent to about 18 cc. per kilo body weight per minute. Mean renal plasma flow was 250 cc. per square meter per minute, σ ±61 cc., SE_m ±6.8 cc. Mean flows separately calculated from phenol red and inulin data agreed within one per cent. Mean plasma phenol red extraction (E_p) was 48.5 per cent, σ ±9.7, SE_m ±1.1. Mean plasma inulin extraction was 29.7 per cent, σ ±7.7, SE_m ±0.87. Mean plasma phenol red clearance was 118.2 cc. per square meter per minute, σ ±25.5, SE_m ±2.91. Mean plasma inulin clearance was 69 cc. per square meter per minute, σ ±14.8, SE_m ±1.7. The mean phenol red to inulin clearance ratio was 1.71.

Although deviations from the means are often wide, the variability of the observations in each experiment is reduced nearly by half by grouping the

data into averages of three or more periods. The range of variation is further reduced in that the experimental data are compared with the control data of the same animal on the same day (tables 2, 3, 4).

Phenol red and inulin extraction percentages and clearances were examined statistically with regard to the correlation of these functions with calculated renal plasma flow.[3] The results of this examination were tab-

TABLE 2

Effect of renin

NO.	PLASMA FLOW		E_p		CLEARANCE		P.R.C. / I.C.	B.P.	SKIN T.	RENIN
	Ph. R.	In.	Ph. R.	In.	Ph. R.	In.				
										cc./min.
1	−46	−44	0	+47	−46	−16	−35	+30	+1.4	0.034
2	−30	−31	+3	+23	−24	−16	−11		0	0.064
3	−35	−22	0	+27	−25	+3	−27	+24	−0.6	0.048
4	−49	−49	−7	+48	−52	−24	−36	+40	−0.6	0.055
5	−25	−28	+13	+58	−16	+14	−27	+24	+0.2	0.085
6	−47	−43	−3	+108	−45	+7	−41	+18	0	0.060
7	−34	−31	+3	+41	−31	0	−31	+40	+0.1	0.080
8	−28	−35	+14	+50	−22	+1	−23	+11	−0.3	0.060
Mean	−35	−35	+3	+50	−32	−13	−36	+29	0	0.060
9	−20	−23	−34	−32	−52	−38	−22	+38	+0.2	0.060
10	+7	+8	−6	+9	0	+25	−20	+21	−0.4	0.075

The mean effect of renin on renal plasma flow as calculated from phenol red and inulin data, on renal plasma extraction percentages of phenol red and inulin and on renal clearances of phenol red and inulin and their ratio and on mean arterial blood pressure expressed as percentage variation from the mean of the control periods. The effect on skin temperature in degrees C. and the dose of renin in cubic centimeters per minute. Ph.R. = phenol red; In = inulin; P.R.C. = phenol red clearance; I.C. = inulin clearance; E_p = extraction percentage; B.P. = mean arterial pressure; T = temperature Centigrade.

[3] The following note is supplied by Mr. K. F. Griffith of Eli Lilly and Company to whom we are indebted for the statistical analysis.

"Pearsonian coefficients of correlation have been obtained for these data by correlating the following two series:

1. Averages of the normal clearance or extraction data falling within ±2 times the Standard Error of their own means $\left(\text{where } S.E._m = \frac{\text{sum of } d^2}{n\ (n-1)}\right)$ at each point of plasma flow when the latter is arranged in intervals of 25 cc. each; against

2. Mid-points of the 25 cc. intervals of plasma flow. For experimental purposes, the median values, instead of the average of data falling within ±2 $S.E._m$, of the flow and clearance percentages have also been similarly correlated against plasma flow. Results are given in table 1, values for b (slope of regression line) having been obtained in standard manner where b = Coefficient of Correlation $\times \frac{S.D._y}{S.D._x}$, $S.D._y = \frac{\text{Sum of } d_y^2}{n}$ and $S.D._x = \frac{\text{Sum of } d_x^2}{n}$."

ulated in table 1. Renal plasma clearances vary directly with renal plasma flow. Renal plasma extractions show satisfactory correlation and vary inversely with renal plasma flow. Regression coefficients calculated from these data were used in constructing the lines drawn through the data of control periods (fig. 1). These lines were extended into the regions of very high and very low plasma flows as reference points for the interpretation of experimental changes.

2. *Effect of renin.* Renal plasma flow and plasma phenol red clearances fell sharply in 8 of 10 experiments in which renin was given, while inulin clearance was relatively or even absolutely increased because of increased inulin extraction (table 2). The fall of renal plasma flow in one experiment (no. 9) was associated with decreased phenol red and inulin clearance and inulin extraction percentage. Arterial pressure was increased in all experiments. Skin temperature was not consistently altered. Diuresis was observed, but was not quantitatively significant because of the presence of sulphate in the infusion fluid.

The results of single experimental periods are plotted in figure 1. Inulin clearances and extraction percentages lie well above the extended regression curves in most instances. There are four periods (expt. 9) in which they lie well below the lines and a few (from expt. 10 in which renal blood flow was not affected) in which they lie near the lines. Glomerular filtration was therefore usually increased as the result of renin infusion to a greater extent than would be calculated from the regression coefficients of normal inulin clearance and extraction with the exception of a few periods in which the reverse or no effect occurred.

Extractions and clearances of phenol red during infusion of renin are distributed evenly about the extended regression curves, although they tend to be relatively decreased at very low levels of plasma flow.

3. *Effect of pitressin.* Infusion of pitressin resulted in moderate or considerable increases of renal plasma flow in 5 of 11 experiments (table 3). Phenol red and inulin clearances and extraction percentages varied much as with normal variations of flow (fig. 1). However, the data include a decrease in the average ratio of phenol red to inulin clearance, the result of slightly decreased extraction of phenol red. Decreased plasma flows were observed in four experiments, in all of which inulin extraction percentages and clearances were relatively increased.

Skin temperature fell about 1°C. Heart rate slowed to about 60 beats per minute. Arterial pressure was first taken 20-30 minutes after the infusion had started and was not consistently altered at this or subsequent observations. Retching, sometimes vomiting and defecation, usually occurred during the first half hour of the infusion. Hematemesis was observed once. Urine flow increased slightly.

4. *Effect of atropine.* Intravenous administration of single doses of

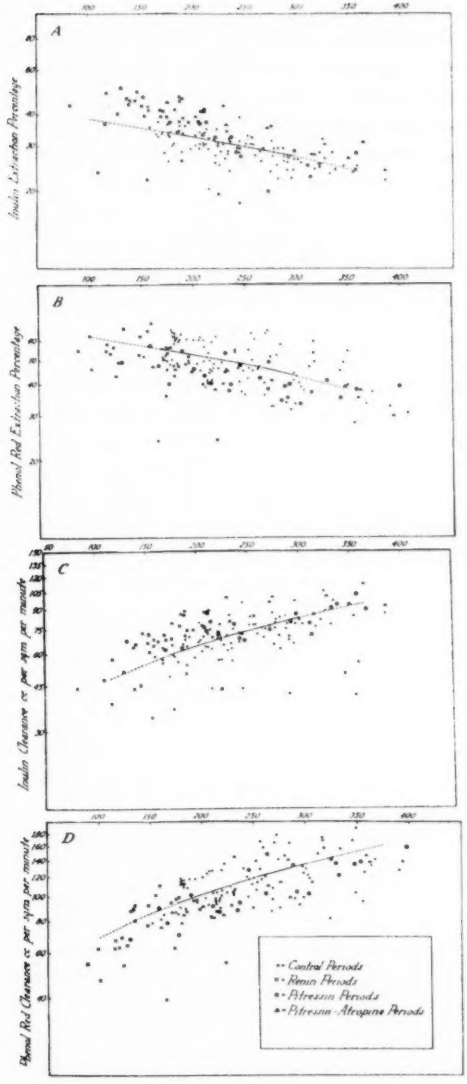

Fig. 1. The relations of renal plasma flow in cubic centimeters per square meter per minute to *a*, inulin extraction percentage; *b*, phenol red extraction percentage; *c*, inulin clearance, and *d*, phenol red clearance in single control and experimental periods. Continuous line is drawn from the regression coefficients calculated from control data and is extended by the broken line into regions of abnormally high and low flow as a point of reference.

0.065 mgm. of atropine during the infusion of pitressin had no consistent effect on renal plasma flow, phenol red extraction percentages or clearances (table 4). Inulin extraction percentages and clearances increased markedly in 5 of 7 experiments. In two experiments renal blood flow increased and inulin extraction was slightly decreased. Blood pressure increased an average of 40 per cent over the level existing during the administration of

TABLE 3

Effect of pitressin

NO.	PLASMA FLOW		E_p		CLEARANCE		P.R.C. / I.C.	B.P.	PULSE	SKIN T.	PITRESSIN
	Ph. R.	In.	Ph. R.	In.	Ph. R.	In.					
											cc./min.
1	+90	+66	−45	−31	+4	+2	−7	−6	−49	−0.7	0.0016
2	+64	+35	−41	−4	+0.5	+34	−35	+5	−47	−0.9	0.0019
3	+16	+23	−28	−10	−11	+11	−19	−12	−45	−0.9	0.0020
4	+75	+53	−32	−24	+8	+15	−7	+13	−48	−1.2	0.0013
5	+33	+37	−9	+7	+37	+43	−13	+7	−46	−1.3	0.0014
6	−22	−29	+11	+60	−23	+16	−30		−54	−1.5	0.0010
7	−46	−40	+62	+91	−18	−7	−12	−7	−62	−1.7	0.005
8	−17	−22	+2	+20	−20	−5	−6	+3	−36	−1.2	0.0014
9	−12	−12	−5	+19	−17	+9	−19	0	−53	−1.0	0.0014
10	0	+3	0	−12	+12	−9	+3			−0.2	0.0009
11	−5	−5	−3	−10	−3	−6	+4	+19		−0.4	0.0004

TABLE 4

Effect of pitressin and atropine

NO.	PLASMA FLOW		E_p		CLEARANCE		P.R.C. / I.C.	B.P.	PULSE	SKIN T.	PITRESSIN	ATROPINE
	Ph. R.	In.	Ph. R.	In.	Ph. R.	In.						
											cc./min.	*mgm.*
1	−25	−13	+39	+51	+18	+3	−10	+40	+240	+0.4	0.0016	0.098
2	+23	+18	−13	−4	+3	+9	−6	+23	+190	0	0.0019	0.065
3	+7	+4	−0	+16	+8	+23	−13	+42	+200	+0.3	0.0020	0.065
4	−38	−25	−11	+35	−39	+3	−42	+66	+240	0	0.0013	0.065
5	+22	+14	−17	−8	0	+3	−3	+67	+200		0.0014	0.065
8	−24	−18	−18	+39	−8	+12	−19	+37	+200	0	0.0014	0.065
9	0	+10	−2	+11	+1	+16	−19	+34	+300	−0.6	0.0014	0.065

pitressin alone. Heart rate more than doubled. The effects on inulin clearance and extraction and blood pressure persisted more than 40 minutes after the administration of atropine.

DISCUSSION. 1. *Control periods.* The values for renal blood flow, plasma phenol red and inulin extraction percentages and clearances during control periods agree in the main with those reported in the literature by Van Slyke, Rhoads, Hiller and Alving (1934), Sheehan (1936), and Mason,

Blalock and Harrison (1937). The average inulin extraction in our series is higher than that found by Van Slyke, Hiller and Miller (1935) but agrees with that calculated by Smith (1938) from the data of Shannon (1935).

Increased phenol red extraction with decreased renal blood flow has been suggested as a probability by Chasis, Ranges, Goldring and Smith (1938). Our data bear out this suggestion. The mechanism of the apparent inverse relation of phenol red extraction and renal blood flow may lie (Smith, 1939) in *1*, increased diffusion from the peritubular capillary bed at lower levels of blood flow; *2*, changes in the capacity of the tubular mechanism of phenol red transport; *3*, the existence of unsuspected arterio-venous shunts in the kidney which exclude the tubules (Spanner, 1938).

An inverse relation of inulin extraction and renal blood flow was predicted by the same authors from observations on normal human beings. They suggested that "an increase or decrease in renal blood flow is accompanied by an inverse change in filtration pressure" so that "filtration rate tends to be independent of renal blood flow". Our findings have, to some extent, confirmed this belief. The mechanism may lie in *1*, changes of intraglomerular pressure; *2*, more complete diffusion of inulin through the glomerular membrane; *3*, the existence of arterio-venous shunts which exclude the glomeruli. As with phenol red, analysis of these possibilities can only be made as more data are obtained.

2. *The action of renin on the kidneys.* Merrill, Williams and Harrison (1938) found in most of their experiments on anesthetized dogs increased blood pressure and renal volume and decreased renal blood flow after single injections of an extract of renal cortex containing the renal pressor substance renin. Others, Hessel (1938), Friedman, Abramson and Marx (1938) found that renin caused in anesthetized dogs a drop of limb volume and usually a rise in renal volume preceded by a fall. Renin has also been shown to increase perfusion pressure and reduce flow through isolated kidneys by Hessel (1938), Friedman, Abramson and Marx (1938). Increased renal volume in association with decreased renal blood flow is usually interpreted as the result of constriction of efferent glomerular arterioles (Richards and Plant, 1922).

The interpretation of changes in inulin clearance or filtration rates with reference to simultaneously observed phenol red clearances has been reviewed elsewhere by Smith, Goldring and Chasis (1938). Briefly, a relative increase of inulin clearance in the presence of decreased renal blood flow and phenol red clearance may be taken as evidence for efferent arteriolar constriction. In our experiments renal blood flow and phenol red clearance both fell about 35 per cent, while inulin clearance fell an average of 13 per cent, and in some cases was increased. Inulin extraction was increased 50 per cent. The fall of renal blood flow and phenol red clearance suggest renal vasoconstriction, while the relative increase of

inulin clearance and the increased extraction of inulin are evidences of increased intraglomerular pressure caused either by increased arterial pressure or by efferent arteriolar vasoconstriction. Although the effect of increased arterial pressure probably shares in the action of renin on the kidneys, our results with pitressin-atropine in which there was a more marked increase of arterial pressure and a smaller change in filtration, suggest that the major action of renin is one of efferent vasoconstriction. A selective efferent vasoconstrictor action of renin is unlikely because of its known effects on systemic arterioles generally. In one experiment (no. 9) the principal renal vasoconstriction seems to have been pre-glomerular. Consequently, it is probable that afferent vasoconstriction was present in the experiments in which filtration was increased, but renin acted more powerfully on the efferent than the afferent arterioles.

Renin does not appear to have a specific effect upon the extraction of phenol red except at very low levels of renal blood flow, where phenol red extraction falls off below that predicted from the extended regression curve.

Renin, unlike other pressor substances, is apparently characterized by its lack of effect on skin temperature (Landis, Montgomery and Sparkman, 1938). This observation is confirmed in our experiments.

The increasing ineffectiveness of repeated doses of renin has been repeatedly observed with single intravenous injections in dogs. However, arterial pressure remained elevated throughout the periods of more than one hour in which renin was slowly infused in our experiments.

3. *The effect of pitressin.* Richards and Plant (1922) found that pituitrin caused a fall of renal blood flow and increased renal volume in anesthetized dogs and rabbits, although it did not have this effect on anesthetized cats. Jannsen and Rein (1928) noted inconstant changes in dogs given anti-diuretic doses of a fresh extract of posterior pituitary lobe. Handovsky and Samaan (1937) found that intravenous doses of 0.0004 to 0.05 cc. per kilo body weight of pituitrin caused definite reduction of renal blood flow in conscious dogs. Walker, Schmidt, Elsom and Johnston (1937) after subcutaneous injection of pituitrin in dogs observed some fall at the onset of anti-diuresis, but that blood flow remained high during anti-diuresis and actually increased in three experiments. Geiling, Herrick and Essex (1934) noted in dogs given pitressin, increased renal blood flow amounting to 200–300 per cent in 3 or 4 experiments (Herrick, 1938).

Our data differ from those reported in that they were derived from experiments in which conscious dogs were given intravenous injections of pitressin over long periods of time. The characteristic initial effects of intravenous pitressin on blood pressure and heart rate were not recorded. Renal blood flow increased in 5 experiments. We would explain this increase as the result of the distribution of blood away from areas of

vasoconstriction (Geiling, Herrick and Essex, 1934), had we not observed decreased renal blood flow in 4 experiments done under similar conditions and with equal reduction of skin temperature. The increased filtration rate observed during the periods of decreased renal flow suggest constriction of the renal efferent arterioles although it is not clear why this constriction was not always present.

4. *Effect of atropine.* The injection of atropine immediately after the injection of pitressin caused increases of pulse rate and blood pressure (Gruber and Kountz, 1930). The rise of arterial pressure is believed to be due to increased cardiac output per unit of time, which is attributed to the release by atropine of the vagal component of the cardiac slowing due to pitressin (Gruber and Kountz, 1930). This observation on the pressor effect of pitressin-atropine has been extended in our experiments to the continuous infusion of pitressin and offers a means of producing sustained hypertension in conscious animals.

The independence of renal blood flow and systemic arterial pressure (Page, 1934), (Hartmann, Ørskov and Rein, 1936) is also shown in these experiments.

The increased inulin extraction observed in most of these experiments is probably due to increased intraglomerular pressure resulting from the rise of systemic arterial pressure.

SUMMARY. 1. A modification is described of the Rhoads method of making the renal vein accessible by explanting the kidney in dogs.

2. An analysis was made of the relations of phenol red and inulin extraction percentages and clearances to calculated renal plasma flow. Phenol red and inulin clearances varied directly with renal plasma flow. Phenol red and inulin extraction precentages varied inversely with renal plasma flow under normal conditions.

3. The slow intravenous infusion of renin in conscious dogs results in decreased phenol red clearances while inulin clearances are relatively increased because of increased inulin extraction. Calculated renal blood flow is greatly decreased. Arterial blood pressure rises while skin temperature is not affected.

4. The slow intravenous infusion of pitressin results in some experiments in increased and in others in decreased phenol red and inulin clearances and calculated renal blood flow. Inulin extraction is increased in those experiments in which renal blood flow falls. Arterial pressure is not affected. Heart rate and skin temperature are decreased.

5. Injection of atropine during the infusion of pitressin results in increased inulin extraction in most instances. Phenol red clearances and calculated renal plasma flow are not constantly affected. Heart rate and arterial blood pressure are greatly increased. Skin temperature is not affected.

CONCLUSIONS

1. The slow intravenous infusion of renin results in increased arterial pressure, renal vasoconstriction and marked reduction of renal blood flow. The glomerular efferent arterioles appear to be the sites of the most marked vasoconstriction. There is no specific effect on the tubular mechanism of phenol red excretion. Decreased renal blood flow and efferent arteriolar constriction are present in essential hypertension in human beings (Smith, Goldring, Chasis and Ranges, 1938). The similarity between the hemodynamics of the kidneys in patients with essential hypertension and in the kidneys of dogs following injection of renin is evident.

2. The slow infusion of pitressin may result in increased or decreased renal blood flow without definite effects on arterial pressure during the experimental period. The absence of increased arterial pressure in the presence of decreased renal blood flow and efferent arteriolar constriction due to pitressin does not exclude the possibility of a secondary renin action during prolonged infusions, since the onset of experimental renal hypertension due to constriction of the renal artery is usually delayed for 24 hours or more.

3. Increased arterial pressure apparently of cardiac origin may be produced by the injection of atropine during the infusion of pitressin. The arterial hypertension results in increased glomerular filtration but has no characteristic effect on renal blood flow.

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THE RELATION OF THE ADRENAL CORTEX TO THE MALE REPRODUCTIVE SYSTEM

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The best available evidence indicates that the adult adrenal cortex does not, except under rare pathological conditions, exert any androgenic activity (5). However, several lines of evidence have suggested that a specialized tissue in the adrenal existing during early life may play a part in influencing the development of the reproductive system. It was the purpose of the present study to investigate to what extent this is true, utilizing the rat and mouse as experimental animals. The adrenals of these two species are characterized by the presence, during early periods of life, of a zone of tissue the growth and development of which are allegedly related to reproductive activity.

The development of the male reproductive system in the absence of the adrenal. In order to determine if the normal adrenal gland exerts any androgenic function, a series of mice and rats were castrated or subjected to castration and adrenalectomy. The latter were maintained in good condition by the oral administration of cortical hormone[1] prepared as described elsewhere (5). Castration was performed in order to stimulate the growth of those regions of the adrenal cortex (x-zone, "juvenile" cortex) which have been assumed to exert an androgenic function. In preparing the reproductive tract for microscopic study, the urethra with its attached glands was removed and fixed for 24 hours in 10 per cent formaldehyde. The tissues, after dehydration, were embedded in paraffin at 50°C. and cut serially at 15 μ. In the case of the adult rats every tenth section, in the young rats every seventh, and in the young mice every fifth section was mounted in balsam after staining with hematoxylin and eosin. The diameter of the tubules and height of the cells were determined with an ocular micrometer at magnifications of 21 and 97 times, respectively.

In table 1 are recorded the cell height and tubule diameter of the ventral

[1] We are indebted to Dr. William A. Feirer and to Mr. Robert Fox for a generous supply of adrenal glands and to Dr. Ernest Blanchard for some of the charcoal adsorbate used in the present study.

and dorsal prostates,[2] and the tubule diameter of Cowper's gland in normal, in castrated, and in castrated-adrenalectomized animals. The results show that the absence of the adrenal gland (when replacement therapy is adequate) is without effect on the development of these glands at least as far as cell height and tubule diameter are concerned. The variations observed in the table are within the limits of normal variability. The

TABLE 1

The effect of castration and of castration and adrenalectomy on the development of the prostate gland and of Cowper's gland of the rat and mouse

Except for the adult rats, the operations were performed at two weeks of age and the animals sacrificed at the time indicated in the second column. The adult animals were sacrificed two weeks following the operations. The microscopic values are averages of measurements on three animals; the gross values are based on one animal in each series.

SPECIES	AGE		SIZE OF					RELATIVE VOLUME OF	
			Ventral prostate		Dorsal prostate		Cowper's gland	Ventral and dorsal prostate	Cowper's gland
			Tubules	Cells	Tubules	Cells	Tubules		
	weeks		*micra*	*micra*	*micra*	*micra*	*micra*	*per cent*	*per cent*
Rat	3	Unoperated control	71	12	57	9	45	100	100
Rat	3	Castrated	53	8	45	9	34	86	90
Rat	3	Castrate-adrenalectomized	54	9	50	7	31	68	86
Rat	4	Unoperated control	75	11	58	10	39	100	100
Rat	4	Castrated	44	9	47	9	27	32	32
Rat	4	Castrate-adrenalectomized	48	8	44	9	29	36	33
Rat	Adult	Unoperated control	97	8	120	7	49		
Rat	Adult	Castrated	50	6	44	5	32		
Rat	Adult	Castrate-adrenalectomized	44	6	38	5	28		
Mouse	3	Unoperated control	40	7	36	7	28	100	100
Mouse	3	Castrated	40	6	35	7	28	82	96
Mouse	3	Castrate-adrenalectomized	43	7	36	7	27	86	87
Mouse	5	Unoperated control	87	10	74	9	53	100	100
Mouse	5	Castrated	43	7	35	6	25	38	16
Mouse	5	Castrate-adrenalectomized	41	6	38	6	28	35	37

young rats and mice were adrenalectomized at a period when their adrenals contain the "juvenile" cells and the x-zone, respectively (8). The results

[2] That portion of the prostate gland which we have designated in the tables as ventral prostate corresponds to the anterior lobe of Walker, the intermediate lobe of Engle, the Prostata III of Rietschel, and the middle lobe of Moore, Price and Gallagher. We have designated as the dorsal prostate that part of the gland designated by Walker and by Moore, Price and Gallagher as the posterior lobe, by Engle as the distal lobe and by Rietschel as Prostata II. (*cf.* van der Woerd (21).)

of table 1 indicate that no androgenic function is exerted by these tissues. It may be noted that the cell heights and tubule diameters of normal rats as found by us do not agree with the values reported by Price (17). This discrepancy is due to the fact that Price limited her measurements to the largest units while ours represent the averages of cells of all sizes.

As the size of a cell or the diameter of the component tubules of an organ in separate sections need not necessarily be indicative of its functional state, we have made a careful cytological study of the accessory reproductive organs of our experimental animals. The cells of the prostate glands of the animals subjected to both castration and adrenalectomy did not differ perceptibly from those which were castrated only. In both series there was some decrease in the stainability of the cytoplasm of the glandular cells. Two weeks following operation in rats or three weeks in mice, the prostates of both groups of experimental animals were in the atrophic state characteristic of castration. The villous processes were absent and there was a marked decrease in the volume of the cytoplasm which was barely stainable. The "light" areas were completely absent. The cells in many tubules formed layers two or three cells deep as compared with the unicellular thickness of the normal gland. There was also a great increase in the amount of the intertubular connective tissue. We have confirmed the delayed atrophy of both the ventral and dorsal prostate of the rat and mouse following castration as described by Price (17) and confirmed by Howard (8) for the ventral lobe in young rats. Our results obviously preclude this effect as being due to any androgenic action by the adrenal. Wiesner (20), in studying other accessory structures in the developing rat (glans penis, seminal vesicle, and coagulating gland) found that these organs also show a decreased rate of growth and a reduction of the growth limit as early as one or, in one case, two weeks after castration performed soon after birth. Thus all the accessory glands appear to have an "innate" potentiality for growth.

The appearance of Cowper's glands and the seminal vesicles also did not differ in the castrate-adrenalectomized animals as compared with those castrated but with intact adrenals (6). In both series of animals there were typical indications of an increase in the size of the central lumen, a decrease in the amount of basophilia of the cytoplasmic contents, a decrease in cell height, the presence of disorganized tubules, and a decrease in the number of terminal tubules in Cowper's glands. These differences were much more prominent in the young animals kept for the longer time-interval after the operations than in those kept for only one week following the operations. In the latter group, the effects were barely noticeable in the mouse, and only slightly more prominent in the rat. Cowper's gland thus appears to show the same delayed atrophy after castration that takes place in other accessory organs of very young rats.

We have also studied the effects of castration and of castration and adrenalectomy on the relative volumes of the glands (see table 1). The relative volumes of the organs were determined by projection with an Edinger apparatus on a good grade of paper, which was cut out and weighed to ± 0.1 mgm. These results are in accord with the conclusions already drawn from the study of the cell size and tubule diameter and of the cytology of the accessory reproductive organs.

The effect of the adrenal cortical hormone on the reproductive system. It may be objected to the conclusions reached in the preceding section that the replacement therapy administered to the adrenalectomized animals contains not only the cortical hormone which is essential for life but also an androgen which serves to replace the loss of androgenic tissue assumed to be present in the adrenal. A number of workers (5, 9, 10, 19) have demonstrated the absence of any stimulating effects of the cortical hormone on the size of the testes of normal animals. To determine more accurately

TABLE 2

The effects of the administration of the adrenal cortical hormone on the development of the tubules of the prostate glands and of Cowper's gland of castrated mice

Castration was performed at two weeks of age and the animals were treated with two rat units of adrenal cortical hormone daily until sacrificed at the age indicated in the first column. The diameter of the tubule is expressed in micra.

AGE		VENTRAL PROSTATE	DORSAL PROSTATE	COWPER'S GLAND
weeks				
7	Controls	38	31	23
7	Treated	37	33	25
8	Controls	43	33	27
8	Treated	38	34	28

if the hormone exerts any influence on the reproductive system, histological studies similar to those recorded in table 1 were carried out on castrated mice, to some of which were administered two rat units of the same adrenal cortical preparation as was used in the other experiments of this study. This dose represents several times the amount of hormone necessary for maintaining normal growth in adrenalectomized mice and thus should give the effects of moderate overdosage. As shown in table 2, there was no androgenic effect elicited by the therapy. This result validates the conclusions drawn from the experiments of table 1.

The androgenic activity of adrenal extracts. Practically all recent writers have quoted Reichstein's isolation (25) of an androgenic substance (adrenosterone) from adrenal extracts as evidence of an androgenic activity by the normal adrenal. Such a conclusion, however, is scarcely justifiable. The amount of adrenosterone obtainable from the adrenals is so infinites-

imally small that one must question the physiological significance of the occurrence of this substance in the adrenals. Of even greater importance is the ease with which adrenosterone can be derived by a hydrolytic process (the reversal of an ordinary aldol condensation) from crystalline compounds found in adrenal extracts (12). Adrenosterone probably is derived by changes occurring in the glandular extracts as a result of chemical manipulation and it is unjustifiable to attribute androgenic activity to the normal adrenal on the basis of the occurrence of this compound in such extracts. The demonstration of androgenic activity in crude adrenal extracts by Parkes (15) and by Pottenger and Simonsen (16) is explicable on the same basis. Hodler's (7) induction of androgenic effects (growth of clitoris) by injection of adrenal tissue into female guinea pigs is probably due to the deleterious effect of such injections on the ovary. Ovariectomy in this animal induces similar androgenic effects.

In view of the ease with which steroid compounds may be altered chemically and the diversity of the compounds thus formed which may exert androgenic action, it is unjustifiable to draw conclusions about the normal physiological function of this gland from the effects of crude glandular extracts of the adrenal. We have attempted to demonstrate androgenic activity in extracts prepared from adrenal glands in which such activity might be anticipated, viz: 1, fetal pig;[3] 2, fetal and newborn human,[4] and 3, x-zone bearing adrenals of mice. To avoid the possibility of degradation products being formed, only fresh glands were utilized and the method of extraction that was employed avoided any violent chemical procedure. The fresh glands were ground into ten volumes of neutral acetone. After standing overnight in the ice chest the mixture was passed through a sintered glass filter and the glandular residues reëxtracted twice with absolute ethyl alcohol. The combined acetone and alcohol extracts were reduced *in vacuo* at 35°C. and the remaining residue dissolved in sesame oil. The extract thus obtained, which would be expected to contain any androgen originally present in the glands, was tested by injection into the castrate rat, by inunction into the comb, and by injection into the white Leghorn capon. In all cases the results of these experiments indicated the absence of any detectable amount of androgen.

One may argue that the amounts of material available for the preparation of our extracts (10 to 20 grams) were insufficient to elicit any androgenic activity. Although they are inconclusive by themselves, the experiments demonstrate the absence of any *considerable* amount of androgen in the adrenal. They cast doubt upon the physiological significance of results

[3] We are indebted to Dr. Louis B. Flexner for kindly placing these adrenals at our disposal.

[4] Some of the human glands were kindly furnished by Dr. W. H. Carnes and Dr. F. B. Kindell.

obtained by the use of extracts derived through complex chemical manipulation of inordinate quantities of glands.

Discussion. The results of the present study demonstrate that the transient zones present in the adrenals of mice and rats in which androgenic activity has been assumed to reside, do not exert any demonstrable effect on the development of the male reproductive system. Comparable experiments demonstrate the absence of any effect of this tissue on female reproductive activity (5). Deanesly (4) has demonstrated that in pituitary-deficient dwarf mice the x-zone is absent in spite of normal reproductive activity. Leblond and Gardner (11) concluded that the x-zone of the mouse does not differ functionally from the rest of the cortex. These workers base their conclusion on the unjustifiable assumption that the ascorbic acid content of this tissue is an indicator of its function. We are elsewhere reporting experiments which demonstrate that the cells of the x-zone behave like the zona reticularis in other animals. Masui and Tamura (13) who first observed the x-zone actually considered it as the zona reticularis of the mouse adrenal.

Despite many observations which can be explained as in conformity with a postulated androgenic function of the adrenal gland, it is possible to explain these phenomena satisfactorily without making such an assumption. Thus the stimulation of growth of the prostate and the induction of estrus in young castrated and spayed rats by the adrenotropic extracts of the hypophysis (3) may be explained as a direct effect of the extract on the prostate rather than as an indirect effect elicited through the adrenals. In this connection it is worthy of note that the prostate is capable of growth in the absence of both testes and "fetal adrenal" tissue. Howard's (8) association of this growth of the prostate with certain cells in the rat's adrenal is obviously untenable on the basis of the results of the present study.

The conclusion that the normal adrenal cortex is devoid of androgenic activity presents no difficulty in explaining the well-known androgenic effects observed pathologically. These rare pathological occurrences (22) may be attributed either to the inclusion of embryonic testicular tissue or to a metabolic dysfunction in which some part of the cortex elaborates an androgenic sterol chemically related to the cortical hormone. It is possible that both of these mechanisms may be involved in the causation of the adrenogenital syndrome. The presence of embryonic testicular tissue in the adrenal may be anticipated from embryological grounds. The occasional occurrence of testicular tissue in the adrenal capsule (1) as well as of adrenal tissue in the testis (2) is evidence of this possible developmental disturbance. Such displaced tissue might give rise to those malignant forms of the adreno-genital syndrome which occur in children. On the other hand, the more benign hyperplasias which give rise to pseudo-

hermaphroditism and the adreno-genital syndrome in adults might be due to a metabolic disorder of the cell which results in the formation of an androgen instead of the chemically related cortical hormone.

SUMMARY

The development of the secondary reproductive organs of male rats and mice were compared in normal, in castrated, and in castrate-adrenalectomized rats and mice maintained on adequate doses of cortical hormone. The results indicate that the adrenal gland does not exert an androgenic function in the normal animal. Neither the "juvenile" cells of the rat nor the x-zone of the mouse normally exert an androgenic function.

The adrenal cortical hormone was found to exert no androgenic effects on the development of the ventral and dorsal prostate glands, of Cowper's gland, or of the seminal vesicles of normal or castrated mice and rats. Extracts of human or pig fetal adrenal glands and of x-zone bearing glands of mice were also found to be devoid of androgenic activity.

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NUTRITIVE REQUIREMENTS OF YOUNG PIGS[1]

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In these experiments pigs 2 to 23 days of age have been raised on artificial diets under the conditions of an experimental laboratory. It is the object of this report to present observations which have been made regarding their nutritive requirements and to describe an artificial diet on which satisfactory growth has been obtained.

REVIEW OF LITERATURE. According to various data for milk consumption of young pigs, these animals take an amount at birth furnishing 96 to 157 cal. (1), 254 to 329 cal. (2) or even 200 to 400 cal. per kgm. daily (3). By the end of the fourth week the food intake is reduced to 100 to 200 cal. (3), 70 cal. (2, 4) or even as little as 35 to 40 cal. (1). Breirem (5), studying growing pigs of 16 to 19 kgm. weight, arrived at the formula $E = 154.74\ G^{0.569}$, E being the daily maintenance requirement and G the live weight. Few studies have been made in pigs weaned at an early age. Terroine's (6) pigs, weighing 9 to 9.5 kgm. each, grew normally on amounts of cow's milk furnishing about 155 cal., while good growth occurred in the two-day old pigs of Newlander and Jones (7) on 260 cal.

Scholz (3) found that suckling pigs consume 20 to 34 grams of protein per kgm. daily at first and about 7 to 11 grams per kgm. by the third week. Observers who have studied pigs of 20 kgm. weight give 7 grams per kgm. as the optimal amount (8, 9, 10, 11) and state that this decreases gradually to 3 or 4 grams when the pigs have attained weights of 90 kgm. It has been found that proteins of animal origin are better than those derived from vegetables (9, 10). More rapid growth and greater economy of gains is said to occur on rations of high protein content (12).

The composition of sow's milk, according to the values recorded by Newlander and Jones (7), averages 6.24 per cent for protein, 6.84 per cent fat, and 6.43 per cent sugar, while the corresponding values as compiled by Hughes and Hart (13) are 6.22 per cent, 6.77 per cent and 5.00 per cent.

[1] Aided by grants from the National Research Council, the Committee on Scientific Research of the American Medical Association, Parke, Davis and Company, Mead Johnson and Company, and the Fleischmann Laboratories, and carried out as a Coöperative Project with the Bureau of Animal Industry, U. S. Department of Agriculture.

These represent nutritive ratios[2] of 1:3.5 and 1:3.2, respectively. According to Washburn and Jones (14) the most favorable nutritive ratio for growth in young pigs is 1:3 or 4.

The average ash content of sow's milk is 0.97 per cent (13), or 5.6 per cent of the total solids. Of these, 1.2 per cent is Ca and 0.46 per cent P (15). According to Lund (16) the ration for young growing pigs should include 0.7 to 0.9 per cent Ca and 0.6 to 0.7 per cent P. The minimum requirement for P in such animals is 0.27 to 0.30 per cent (17). Dunlop (18) favored a Ca level of 0.45 per cent with a Ca:P ratio of 1:1.3. The ratio recommended by Isaachsen et al. (19) is similar (1:1.22) but Mitchell et al. (20) found the daily requirements of Ca and P were 1.6 mgm. and 7.0 mgm. per kgm. body weight, respectively.

According to Dunlop (21) pigs require 14 to 62 mgm. carotene per 100 lb. ration. This is equivalent to about 13 to 55 micrograms per kgm. The minimum requirement according to Guilbert et al. (22) is 25 to 30 micrograms daily per kgm. body weight. The minimum vitamin A requirement was found by these workers to be 6 to 8 micrograms daily. They considered that at least 5 to 10 times this amount is desirable. There are many reports in the literature concerning avitaminosis A in swine (23, 24, 25, 26).

The pig's requirement for the vitamin B complex has recently received the attention of Chick and her co-workers (27, 28) and of Hughes (29). In both series of experiments the value of nicotinic acid for the pig has been clearly shown and the need for thiamin appears highly probable. These substances were given in amounts of 0.3 to 1.8 mgm. and 17 to 90 micrograms per kgm. body weight daily, respectively. Chick and her associates were unable to conclude whether riboflavin (34 to 140 micrograms per kgm.) is an essential dietary constituent for the pig but Hughes found that this vitamin, given as whey adsorbate, resulted in better appetite, more rapid growth and a more economical increase in body weight. Optimum growth did not occur, however, when thiamin, riboflavin and nicotinic acid were given. Chick et al. showed that two further water-soluble vitamins, contained in filtrate and eluate fractions of liver, are necessary. Hughes found a similar essential in a rice bran filtrate. Dried yeast (4 per cent) contains all the water-soluble dietary essentials (28).

It is generally assumed that the pig does not require the specific inclusion of vitamin C in its diet. Our own observations on this subject will be considered separately (30). No reports concerning the requirement of vitamin E by the pig have been found.

MATERIAL AND METHODS. The pigs were derived from 8 pure bred litters (Poland-China, Duroc-Jersey, Yorkshire) and 5 crossbred litters

[2] Ratio, in calories, of protein to fat plus carbohydrate.

(Landrace, Yorkshire; Landrace, Poland-China; Landrace, Large Black; Duroc-Jersey, Landrace) and were farrowed at the U.S. Animal Husbandry Station, Agricultural Research Center, Beltsville, Md. Observations have been made on a total of 95 animals. Of these 53 were shipped to Baltimore at 2 to 9 days of age, the remainder at 15 to 23 days.

The animals were housed on concrete floors, either in an animal room or out of doors. Shelter and wooden platforms were provided but straw bedding was not used because the pigs were found to eat it. Room temperature was kept at 50 to 60°F. as far as possible. Many of the animals were housed and fed in separate pens.

The basal diet consisted of various proteins, carbohydrates and fats, as will be indicated later, and in different ratios as shown in table 1. This diet was supplemented by a mineral mixture and various vitamins.

TABLE 1

Quantities of protein, fat and carbohydrate in various diets used

DIET DESIGNATION	PROTEIN*	FAT	CARBOHYDRATE	NUTRITIVE RATIO
	per cent	*per cent*	*per cent*	
A	41.2	39.7	19.1	1:2.7
B and C	34.2	39.1	36.6	1:3.6
KUD 1	35.4	27.0	37.6	1:2.8
D, E, and KUD 2 and 3	24.0	13.5	62.5	1:3.9
KUD 4	27.0	32.3	40.7	1:4.2

* The protein is calculated on the basis of the nitrogen content of the casein (and lactalbumin) used.

"KUD" is an abbreviation for "kilo unit diet," a unit representing the amount of food furnishing 156.5 calories. The actual composition of KUD 2, the diet chosen as the most satisfactory, was casein 9.5 grams, lard 4.0 grams, cod liver oil 0.5 gram, sucrose 21.0 grams, salt mixture 2.2 grams, total 37.2 grams.

RESULTS. "*Synthetic milk.*" A fluid which superficially resembles milk was made with the aid of a stirring machine and homogenizer (31) (diets A, D, E, table 1). It was found, however, that pigs only a few days old soon learn to take food as a gruel from a shallow crock or trough and consequently the troublesome procedure of making milk was abandoned.

Types and amounts of protein and carbohydrate, and caloric requirement. Protein formed 24.0 to 41.2 per cent of the diets (table 1) and consisted of casein alone (diets A, B, D and KUD 1, 2, and 4) or casein, 62.5 per cent and lactalbumin, 37.5 per cent (diets C, E and KUD 3). The growth of 12 pigs given the mixture of proteins was no better than that of pigs given casein as the sole source of protein.

Sucrose seemed to be satisfactory as the source of carbohydrate. Neither lactose (diets B and C (2), table 1), dextrin (diet C (4)) nor whey

powder (lactose 72 per cent) (diet C (3)), were superior either as regards growth or in preventing diarrhea.

In figure 1 are compared the growth curves of young pigs given various amounts of cow's milk and of those given an artificial diet (KUD 2) supplemented with yeast. There is little difference between them.

The pigs were given amounts of food furnishing an arbitrarily chosen "kilo unit" (156.5 cal. per kgm. daily) or multiples thereof. Neither in those fed milk nor in pigs given the artificial diet was growth, on the aver-

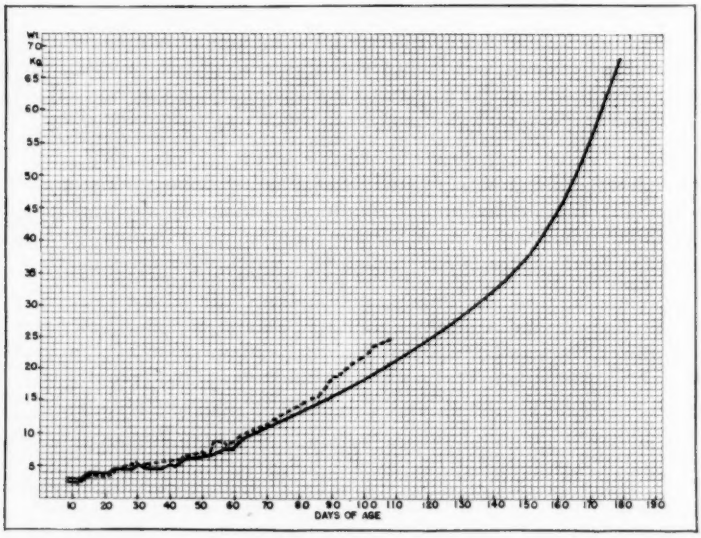

Fig. 1. Growth of 8 pigs weaned at 8 to 24 days of age and given cow's milk, compared with that of 43 pigs weaned at 8 to 18 days and given an artificial diet (KUD 2) supplemented with 3 to 6 grams yeast per kgm. body weight. Three of the pigs fed milk were given 236 cal. per kgm. and 4 of those given the artificial diet received this amount. The remainder were given 157 cal. per kgm.

Interrupted line, cow's milk; continuous line, artificial diet.

age, more rapid when more than one unit was given than when only 156.5 cal. were furnished, as long as an adequate amount of yeast and a suitable mineral mixture were provided for the pigs given the artificial diet. On the other hand, half a unit (78 cal.) was definitely inadequate. Of 4 pigs, litter mates and raised under exactly similar conditions, 2 given 1 unit of diet KUD 2, gained an average of 619 grams daily between the ages of 104 and 171 days whereas 2 given half a unit gained only 309 grams daily during the same time.

Mineral requirement. When an inadequate supply of minerals was

given, even though the diet was presumably satisfactory in other respects, growth was slow or ceased entirely (fig. 2), spontaneous fractures occurred, roentgenograms of the bones showed widespread osteoporosis, and death followed in 10 or 11 weeks. At autopsy the bones were found to be very

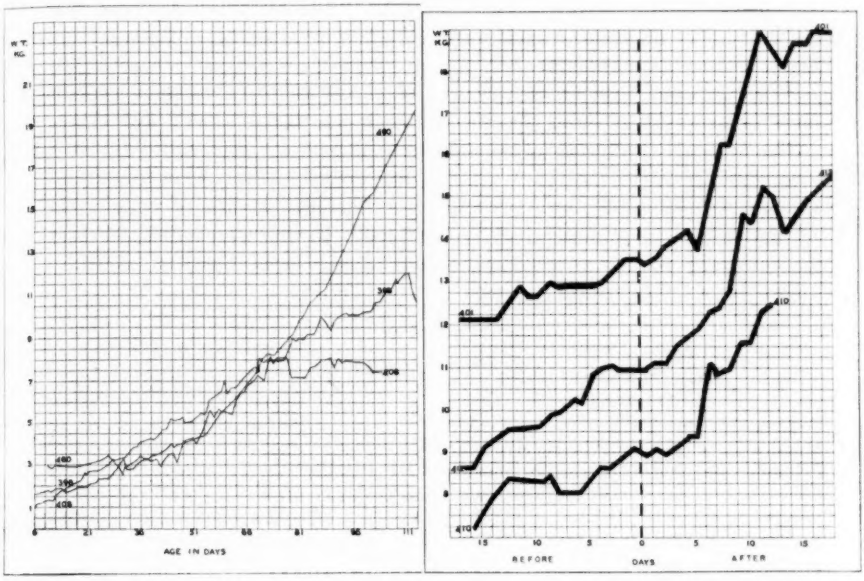

Fig. 2. Comparison of rate of growth in two pigs receiving an inadequate amount of minerals (3–98, 4–08) with that of a pig (4–60) given the salt mixture shown in table 2.

3–98 and 4–08 were weaned at 3 days of age but they were given one quart of cow's milk daily for 39 and 35 days respectively. After this time they received diets very low in minerals (0.03 gram Cowgill salt mixture (32) and 0.15 gram ferric ammonium citrate per 100 grams solids) but adequate in other respects (diets B and C (table 1) supplemented with cod liver oil and yeast).

4–60 was weaned at 9 days of age, received no cow's milk, but was given diet KUD 2 (table 1) supplemented with cod liver oil and yeast.

Fig. 3. Growth rate in 3 young pigs changed at *O* from a diet containing 0.03 per cent Cowgill salt mixture and 0.15 per cent ferric ammonium citrate, to one containing 5.91 per cent of swine salt mixture (table 2).

Pig 4–01 was 115 days of age at the time of the change in minerals, pigs 4–10 and 4–12 were 83 days old.

soft and were easily cut. The ribs of a pig 115 days old (3–98) could be twisted with ease and fractures of 3 of the long bones and of the lumbar spine were found at autopsy. The teeth were worn, chipped and pitted, although they seemed quite hard in comparison to the bones.

Abderhalden (15) recorded values for various minerals in sows' milk. With these as a basis, the salt mixture shown in table 2 was prepared. This imitates the values recorded by Abderhalden and, in addition, includes iodine as well as a much greater quantity of iron than is found in milk. This salt mixture formed a part of diets KUD 1, 2, 3 and 4, 2.2 grams per 35.0 grams of other solids, or 5.91 per cent. The quantities of Ca and P are 0.94 and 0.52 gram, respectively, per 100 grams solids, or 336 and 186 mgm. respectively per kilo unit.

The increased rate of growth following the addition of this mineral mixture was very striking (fig. 3). Growth was accelerated 2 to 9 times.

The findings at autopsy of pigs fed this salt mixture were quite different from those above described. No fractures or dislocations were found and the bones were quite hard, even in an animal which died of pneumonia 13 days after the minerals had been added. In one which died 35 days following the change in minerals, an old fracture was found to be firmly united by a strong callus.

TABLE 2

Swine salt mixture

	grams
Sodium chloride, NaCl	11.71
Magnesium citrate, $Mg_3(C_6H_5O_7)_2 \cdot 14H_2O$	13.27
Primary (monobasic) potassium phosphate KH_2PO_4	4.92
Secondary (dibasic) calcium phosphate $CaHPO_4 \cdot 2H_2O$	39.92
Potassium chloride, KCl	5.85
Ferric citrate, $FeC_6H_5O_7 \cdot 3H_2O$	9.52
Potassium iodide, KI	0.19
Calcium carbonate, $CaCO_3$	14.62
	100.00

Vitamins A, D and E. Vitamins A and D were furnished in butter and in cod liver oil. The last was usually given in doses of 0.5 gram per kilo unit and was added freshly to the diet each day. The variety used (Mead Johnson) furnishes 1800 units A and 175 units D per gram. No signs of deficiency of vitamins A and D were observed in our animals.

Only 2 pigs received *wheat germ oil.* The addition of this oil in the ratio of 1.6 per cent to diet KUD 2 supplemented with yeast or liver extract, was associated with no significant changes. It has been stated that cod liver oil and even lard may contain small amounts of vitamin E (33). Consequently our animals may have been receiving sufficient amounts already.

The B complex. The casein used in these studies was free of water-soluble vitamins, except that given pigs 4–60 and 4–63 to 4–66, inclusive, after they were 140 days old, and that given pigs 4–78 to 5–15, inclusive,

throughout the whole period of observation. Vitamin-free casein was either purchased (Labco, Harris) or acid-washed in our own laboratory by the method described by McCollum et al. (34). The crude casein used was Sheffield "New Process." In all instances when vitamin B-free casein was used, the butter and lard in the food mixture were melted and washed several times in water.

Of the animals receiving the artificial diet, 26 were given 3 grams *yeast*[3] per kgm., 17 received 6 grams. There was no significant difference in the growth rates of the 2 groups (table 3).

TABLE 3

Average daily weight gains of pigs given basal diet (KUD 2) supplemented by various amounts of yeast and one or more synthetic vitamins

QUANTITY OF YEAST	SYNTHETIC VITAMINS	NUMBER OF PIGS	AGE OF PIGS		DAILY WT. GAIN	
			Beginning of expt.	End of expt.	Av.	Range
grams	*micrograms*		*days*	*days*	*grams*	*grams*
1.5–0.1	Thiamin 70–285	5	103 (67–151)	170 (98–245)	93	(86–100)
1.5–0.1	Thiamin 150–300 Riboflavin 60–140	6	82 (67–102)	166 (102–235)	108	(36–200)
1.5–0.5	Thiamin 285 Nicotinic acid 2000	3	69 (67–73)	131 (123–139)	160	(105–270)
1.0–0.2	Thiamin 285 Riboflavin 140 Nicotinic acid 2000	3	68 (68)	167 (131–208)	237	(150–330)
3.0	None	10	84 (83–85)	159 (153–163)	480	(246–700)
6.0	None	6	83 (83)	159 (157–163)	349	(246–445)

All pigs were fed the same number of calories (156.5). The data for quantity of yeast and of the synthetic vitamins refer to the amount given per kilogram body weight daily.

As the quantity of yeast was reduced below 1 gram per kgm., the rate of growth decreased and sometimes ceased entirely (fig. 4). The animals no longer appeared active, clean and well rounded out, and their hair became curled and the skin dirty and scaly. Diarrhea and sometimes vomiting developed. When the yeast was withdrawn entirely and the deficiency became severe, prostration developed suddenly and convulsions occurred. If the reduction in yeast was carried out gradually, chronic deficiency was produced and in addition to these signs of malnutrition, the gait of the animals became impaired. Eventually marked ataxia developed and

[3] "Yeast Foam Tablet Powder," Northwestern Yeast Company, Chicago.

degeneration of the whole sensory neuron (ganglion cells, posterior columns of the spinal cord, and peripheral nerves) occurred (35). In a number of the pigs anemia developed as well (36).

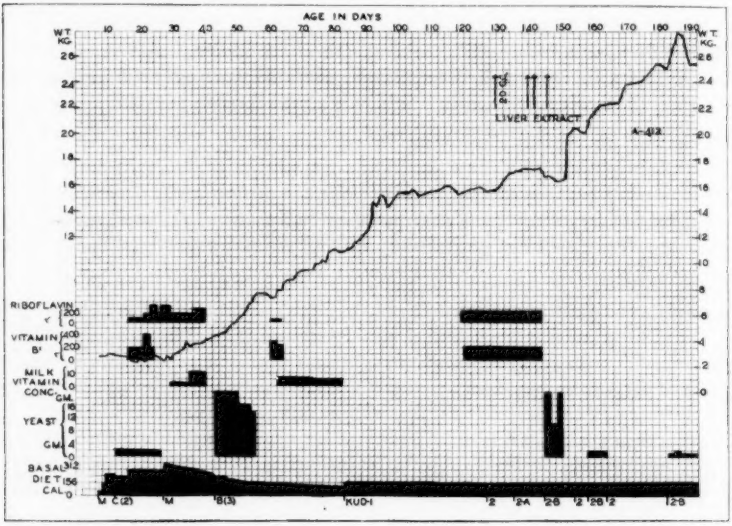

Fig. 4. "Case history" of one of the pigs (4–12) weaned at 6 days of age. Note the sharp gain in weight when sufficient minerals were added (at KUD 1); the failure to continue gaining weight when no supplements were added to the diet of casein, butter fat, sucrose and minerals; the slight improvement following the administration of vitamin B_1 (thiamin chloride), riboflavin, cornmeal (at 2A) and liver extract; and the marked improvement when yeast was added. The animal finally died suddenly after several days of diarrhea.

Quantities given are indicated as calories, grams or micrograms per kgm. daily. The letters and numbers along the base of the chart refer to different types of diet: *M* means cow's milk; *C* (2) consisted of casein, lactalbumin, lactose and butter fat; in *B* (3) lactalbumin was omitted and whey powder was substituted for lactose. These diets were inadequate in mineral content. In those that followed, the mineral mixture shown in table 3 was used (5.91 per cent). KUD 1 and 2 differed in their relative proportions of casein, butter fat and sucrose as shown in table 1. Diet KUD 2 included no supplements; in KUD 2a 15 grams yellow cornmeal were substituted for the same amount of sucrose; in KUD 2b, yeast was used in the quantities shown in the chart.

Vitamin B_1 (thiamin chloride)[4] was given to pigs receiving a diet free of water-soluble vitamins or supplemented with various amounts of yeast. When 3 grams yeast per kgm. were being given, no change was noticed on

[4] A synthetic product furnished by Mead Johnson and Company and the Winthrop Chemical Company.

the administration of B_1. When the quantity of yeast had been reduced as low as 0.5 gram or 0.2 gram per kgm., a substantial increase in the rate of growth and return of appetite followed the intramuscular injection of B_1 in doses equivalent to 70 γ per kgm. daily. Thus in pigs 4–60, 4–64 and 4–65, whose growth for the preceding 29, 21 and 52 days had averaged 11, 0 and 57 grams daily respectively, the administration of B_1 was followed by weight gains of 333, 90 and 146 grams, respectively, in the succeeding 32, 21 and 49 days (fig. 5).

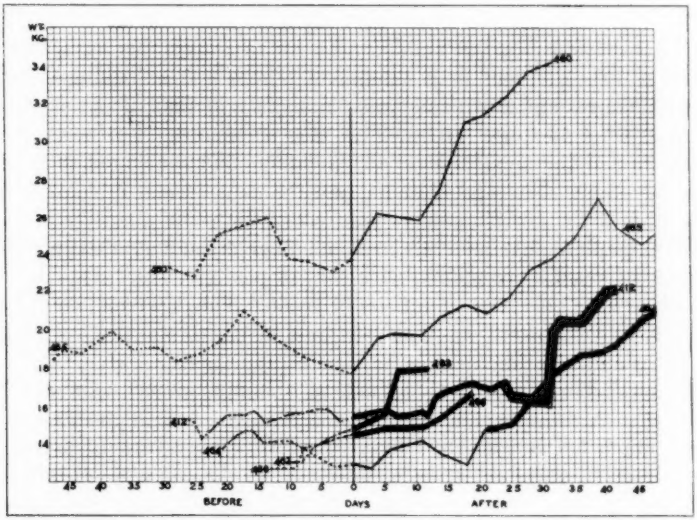

Fig. 5. Growth of 6 young pigs before and after the administration of thiamin and riboflavin.

A narrow single line indicates that thiamin was given; the heavy lines indicate that both riboflavin and thiamin were given; the period during which pig 4–12 received yeast in addition (average of 11 grams per kgm. daily) is underlined.

Pig 4–12 received no yeast whatever until 25 days after thiamin and riboflavin were first given. The remainder of the pigs received 0.5 to 0.1 gram of yeast per kgm. daily.

Riboflavin[5] was given by intramuscular injection to several animals already receiving 3 grams yeast per kgm. No change was observed. When an inadequate amount of yeast was being given, some improvement in growth followed the injection of this substance. Pig 4–64 received riboflavin, in amounts equivalent to 60 γ per kgm. per day, after growth at the rate of 90 grams per day had followed the administration of thiamin

[5] A synthetic product furnished by Merck and Company, Inc., and the Winthrop Chemical Company.

(fig. 5). When the riboflavin was added, growth improved still more, averaging 203 grams in the next 56 days. Pigs 4–63 and 4–66 received both B_1 and riboflavin, in amounts equivalent to 150 γ of the former and 60 γ of the latter per kgm. per day. The growth rates improved 14 and 60 per cent, respectively. In pig 4–12 (figs. 4 and 5) during a period when no yeast whatever was given, B_1 and riboflavin were injected. Modest weight gain, 80 grams per day, followed. The inadequacy of these substances in supplying all the growth factors present in yeast was well shown by this animal. When yeast was given, the average weight gain rose to 400 grams daily.

Nicotinic acid was given in daily amounts of 2 mgm. per kgm. to 6 pigs receiving diets deficient in B vitamins. There was a striking difference between the rate of growth of these animals and that of other pigs not supplied with adequate amounts of this substance. Furthermore, the appearance of the animals receiving nicotinic acid continued to be good, their hair being clean and straight, and the skin normal in color.

In table 3 the growth rate of pigs given the same quantity of carbohydrate, protein, fat, minerals and cod liver oil and gradually reduced amounts of yeast, is compared with that of pigs given the same diet supplemented with adequate quantities of yeast. The pigs receiving the diets deficient in yeast were supplied with the synthetic substances thiamin, riboflavin and nicotinic acid in various combinations.

When riboflavin was given as well as thiamin, growth rate was little better than without it, but the addition of nicotinic acid to thiamin permitted much better growth. That riboflavin may be useful to the pig, even though to a limited extent, is suggested by the still more rapid growth of the pigs given all three vitamins. The failure of thiamin, riboflavin and nicotinic acid to completely replace the growth factors present in yeast is shown by the much better growth of the animals given adequate amounts of yeast. Furthermore, ataxia and changes in the nervous system as well as anemia developed when insufficient yeast was given, even though thiamin, riboflavin and nicotinic acid were supplied (35, 36).

Yellow *cornmeal*, which contains large quantities of vitamin B_6, was given to several pigs receiving deficient diets but no significant changes were observed (fig. 4).

An attempt was made to determine whether vitamins are present in milk which are absent from yeast. A *milk "vitamin concentrate"* (Labco) one gram of which is derived from 97 cc. of milk was used. No advantage from the use of this substance was observed.

No advantage over yeast was observed when *liver extract* was given to 2 pigs as a supplement to diet KUD 2. When 3 grams per kgm. (liver extract, 343, Lilly) were given, growth rate averaged 70 grams per day and their undernourished appearance and scaly and dirty skin and hair did not improve until 10 grams were given daily. Liver extract injected intra-

muscularly in 4 doses of 20 cc. each (Lilly, unconcentrated) relieved the convulsions which developed in pig 4–12 (fig. 4) when it was receiving a diet free of water-soluble vitamins except for thiamin, riboflavin and those present in yellow cornmeal. Some increase in weight followed (70 grams per day) but the weight gain which accompanied the subsequent administration of yeast was much greater (353 grams per day).

Discussion. It is now well recognized that differences exist between various mammals in regard to their nutritive requirements. Although much of the information regarding nutrition which has been derived from studies in rats has been applied successfully to man, an illustration of the confusion which may result from conclusions based exclusively on studies of the rat, is found in the assumption once made that the dermatitis which develops in the rat when it is deprived of the heat-stable portion of the B-complex, is the analogue of human pellagra. These two disorders have been shown to be the result of deficiencies of two quite different factors both of which are present in yeast.

The pig has not been used to a great extent for nutritional studies even though the nutritional requirements of this animal and of man are probably similar in many respects. The latter statement applies particularly to the nutritional factors necessary for normal blood formation. Thus it has been shown that the content of anti-pernicious anemia principle in the liver of the dog is much lower than in that of the pig (37). The liver and stomach of the pig are the chief sources of substances used in the treatment of pernicious anemia.

The artificial diet here described (KUD 2), when supplemented with yeast, permitted satisfactory growth and nutrition. As compared with the development of animals raised on experimental farms and not weaned until 7 to 10 weeks of age (38, 39), growth in our animals was poor in the first few weeks after weaning. Their early weaning and the less satisfactory housing of such animals in a medical school laboratory as compared with that found in modern experimental farms, might account for the differences observed. Furthermore, diarrhea was an almost regular complication when the pigs were weaned. This was controlled by mixing apples, well ground up, with the diet during the first week or more. After 9 to 13 weeks of age the growth of our animals was as good as that observed on experimental farms and sometimes very good growth was observed much earlier.

Casein seems to be adequate as a source of protein. Essential fatty acids are present in lard and in cod liver oil (40). Sucrose was satisfactory as the source of carbohydrate, which is a matter of some importance in studies concerned with vitamin B deficiencies because it has been reported that, unlike lactose or cornstarch, sucrose neither carries with it nor favors the production in the intestine of riboflavin, vitamin B_6 or "filtrate factor" (41).

SUMMARY

1. An artificial diet which permitted good growth in young pigs weaned at 2 to 23 days of age is described.

2. The protein of the diet (24.0 per cent) is derived from casein, the fat (13.5 per cent) from lard, and the carbohydrate (62.5 per cent) from sucrose.

3. An amount furnishing about 157 calories per kgm. body weight daily, is a satisfactory quantity of this diet.

4. A suitable mineral mixture is presented, details of which are given in the text. This mixture imitates the mineral content of sow's milk but includes adequate amounts of iodine and iron as well. When an inadequate supply of minerals was given spontaneous fractures occurred.

5. Vitamins were furnished by giving cod liver oil and yeast, 0.5 gram of the former and 3 or more grams of the latter per kgm. per day.

6. When the quantity of yeast was reduced, impairment of growth followed. In such animals growth was accelerated when thiamin chloride, 70 to 300 γ per kgm., was injected daily. The need for riboflavin, 60 to 140 γ per kgm. daily, was not as clearly demonstrated. The effect of the administration of nicotinic acid, 2 mgm. per kgm. daily, on growth and skin, was very striking.

7. It was clearly evident, however, that the failure of growth associated with the use of inadequate amounts of yeast could not be entirely corrected by the use of synthetic thiamin chloride, riboflavin and nicotinic acid.

8. The necessary growth factors seemed to be more readily available in yeast than in liver extract. No evidence was found that a concentrate of milk vitamins contained growth factors not present in yeast.

The writer wishes to express his appreciation of the helpful advice of Dr. E. V. McCollum, Miss J. Ernestine Becker, Dr. L. Emmett Holt, Jr., and many other associates at the Johns Hopkins University, and of Dr. Samuel Weisberg of the Sealtest Laboratories, who also furnished materials.

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IODOACETIC ACID AND ANAEROBIC MUSCULAR CONTRACTION

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Since the discovery that iodoacetic acid prevents the formation of lactic acid in muscle (Lundsgaard, 1930a) this enzyme poison has been widely used in the study of reactions in muscle extract. From these investigations there has been evolved an explanation of the interrelations between the chemistry of anaerobic contraction and the mechanism of glycolysis. The central position in this schema is occupied by adenosine triphosphate, in that this substance is considered to take part in many of the reactions. The breakdown to adenylic acid and inorganic phosphate is the initial reaction; the inorganic phosphate reacts with glycogen to form hexosemonophosphate, while the adenosine triphosphate is restored by transfer of the phosphate group of phosphocreatine to the adenylic acid. The hexosemonophosphate now reacts with adenosine triphosphate, forming hexosediphosphate and more adenylic acid. The hexosediphosphate is converted to triosephosphate, which undergoes a series of reactions, again involving adenylic acid and adenosine triphosphate. In the course of these transformations lactic acid is the end product, and the phosphocreatine is resynthesized. There is also an alternate mechanism for the initial reaction, in which the inorganic phosphate normally present reacts with glycogen. Summaries of these reaction cycles are given by D. Needham (1937) and Parnas (1937).

The first point in extract reactions which is affected by iodoacetate is the conversion of triosephosphate into its oxidation and reduction products: phosphoglyceric and glycerophosphoric acids. This reaction is completely inhibited by the poison (Embden and Deuticke, 1934). Reaction between phosphocreatine and adenylic acid is partially inhibited (Jacobsen, 1933; Lohmann, 1934), but the conversion of hexosediphosphate into triosephosphate is not affected with respect to either the rate of reaction or the equilibrium point reached (Meyerhof and Lohmann, 1934).

On the basis of the known effects of iodoacetic acid in extract and the reaction-cycle referred to, the initial reaction in the poisoned muscle contracting anaerobically should be the formation of hexosemonophosphate

from either inorganic phosphate or adenosine triphosphate. This should be followed by the formation of the diphosphate and the breakdown of adenosine triphosphate; part of the hexosediphosphate should be immediately converted to triosephosphate. Since it has been found that normal muscle in recovery shows only a very slow conversion of adenylic acid to adenosine triphosphate (Sacks, 1938), and since the iodoacetic acid tends to inhibit this reaction, it should take place very slowly if at all in the poisoned muscle. Phosphocreatine might thus be expected to undergo little change.

However, it is well known that the most prominent reaction taking place in the poisoned muscle is the disappearance of phosphocreatine. This was shown in the earliest experiments of Lundsgaard (1930a), and he was later able to show that this is the first reaction taking place (1934), although it was not definitely established what became of the phosphate groups liberated in the early stages of this breakdown.

The present experiments were undertaken to study more fully the fate of phosphocreatine in anaerobic contraction of muscles of frogs poisoned with iodoacetic acid, and also to study the other changes in phosphorus compounds which take place under these conditions. In this way it might be possible to correlate the findings either with those in normal muscle or with the extract reactions, and thus gain a clearer insight into the mechanisms supplying the energy for muscular activity in the absence of oxygen. The general procedure used was to transect the spinal cord of the frog, inject an adequate amount of iodoacetic acid, and after allowing time for the poison to take effect, stimulate one gastrocnemius muscle while leaving the other one resting. The amount of iodoacetic acid used was 0.11 mgm. per gram of frog, as the sodium salt. Preliminary experiments had shown that this amount was sufficient to cause inhibition of lactic acid formation in contraction without producing too rapid onset of rigor. Under these conditions the muscles remaining connected to the central nervous system pass into rigor at a time when the muscles which have lost their central connections are apparently normal except for their inability to form lactic acid. This spontaneous rigor appeared in the fore legs between 40 and 50 minutes after the injection of the acid. As soon as this took place, one gastrocnemius muscle was frozen with a mixture of solid carbon dioxide and ether, and the other attached to an isometric lever for stimulation. Condenser discharges through a thyratron at the rate of 1 per second were used to stimulate the sciatic nerve.

The first group of muscles was subjected to 20 twitches each. With this amount of activity there was no evidence of fatigue or contracture. A second group of muscles was stimulated to the first sign of contracture, which usually appeared after 75 to 100 twitches. Some fatigue was also present, as the twitch tension developed had decreased to less than half

the initial maximum. The muscles of these two groups were frozen immediately after the cessation of stimulation. In a third group the muscles were stimulated to complete fatigue and loss of indirect irritability. Contracture and rigor were not complete at this point, for some shortening took place in the two minutes following the loss of irritability, and the muscles were not frozen until the maximum shortening had been reached. The conditions during the stimulation period were not strictly anaerobic, but the amount of oxygen present in the muscles could not supply energy for more than one or two twitches.

Analyses for inorganic phosphate, phosphocreatine, the labile phosphate groups of adenosine triphosphate, and hexosemonophosphate formed, were made by the methods used previously (Sacks and Sacks, 1933). The alkaline hydrolysis method of Meyerhof and Lohmann for the determination of triosephosphate was applied to the rigor muscles. Determinations of adenosine triphosphate were not made on these muscles, since the presence of hexosediphosphate makes the acid hydrolysis method invalid. Lundsgaard has shown by other methods (1930b) that practically all the adenosine triphosphate is broken down in the poisoned muscles in rigor. It was established by hydrolysis curves that a large part of the total hexose ester present in the rigor muscles was in the form of diphosphate. It was assumed that the distribution was similar to that obtained by Lundsgaard (1930b) under the same conditions: three-fourths as diphosphate and one-fourth as monophosphate.

The findings in the first group, shown in table 1, are in agreement with those of Lundsgaard (1934): phosphocreatine is decreased, without change in adenosine triphosphate, by an amount of contraction which does not lead to fatigue or contracture. The larger part of the phosphocreatine-P which has been lost is accounted for by hexosemonophosphate formed, while a smaller part has been hydrolyzed to inorganic phosphate. If the hexosemonophosphate formation took place by reaction of glycogen with either inorganic phosphate or that liberated from adenosine triphosphate, the phosphorus distribution in these muscles should be entirely different. This assumes that the same slow resynthesis of adenosine triphosphate occurs in the poisoned muscle that has been found in normal muscle (Sacks, 1938).

Stimulation to the onset of contracture, shown by the second group in table 1, results in the continuation of the breakdown of phosphocreatine, with further accumulation of both hexosemonophosphate and inorganic phosphate. In addition, there is noted here the first evidence of breakdown of adenosine triphosphate. It is impossible to determine whether the phosphate liberated in this breakdown is converted to the inorganic form, or to hexosemonophosphate. If any hexosediphosphate is formed at this stage, the amount is too small to be determined.

The changes taking place from the onset of contracture to the complete development of rigor, shown by the third group in table 1, are concerned

TABLE 1

Phosphorus changes in anaerobic contraction of muscles of frogs poisoned with iodoacetic acid

Values expressed in terms of milligrams per cent of P

RESTING MUSCLE			STIMULATED MUSCLE			DIFFERENCE			
Inorganic	Phosphocreatine	Adenosine triphosphate	Inorganic	Phosphocreatine	Adenosine triphosphate	Inorganic liberated	Phosphocreatine breakdown	Adenosine triphosphate	Hexosemonophosphate formed
			20 twitches						
26	63	32	30	40	33	4	23	1	18
23	58	35	24	44	34	1	14	−1	14
30	58	15	37	43	15	7	15	0	8
16	72	22	37	44	22	21	28	0	7
17	77	25	21	66	28	4	11	3	4
Average						7	18	0	10
			Stimulation to onset of contracture						
20	71	40	35	12	25	15	59	−15	59
19	64	32	43	13	34	24	51	2	35
24	57	36	39	16	34	15	41	−2	28
23	56	35	55	3	27	32	53	−8	29
19	52	35	40	0	28	21	52	−7	38
Average						21	51	−6	38
			Stimulation to complete rigor					Triosephosphate	Total hexosephosphates formed
					Liberated by alkaline hydrolysis				
29	51	35	11	9	0	−18	42	0	85
20	65	29	20	7	0	0	58	0	87
26	45	29	25	3	1	−1	42	1	71
20	51	30	4	6	1	−16	45	1	91
27	54	39	6	9	0	−21	45	0	105
28	39	30	22	4	1	−6	35	1	71
18	55	36	20	11	0	2	44	0	68
Average						−9	44	0	84

with the conversion of the hexosemonophosphate previously formed, into the diphosphate. Any phosphocreatine which was present at the beginning of contracture remains as such in the rigor muscles. Part of the

phosphorus for the formation of the hexosediphosphate is supplied by adenosine triphosphate, as it is in muscle extract, but a rather large amount of inorganic phosphate is also utilized. This latter reaction has not been found to take place in extracts.

No evidence was found for the presence of triosephosphate in the rigor muscles. The small amount indicated in some of the muscles is within the limits of error of the colorimetric method. The amount of phosphate present was so small that it was necessary to add standard phosphate solution to the samples to obtain sufficient depth of color to enable a comparison to be made. Meyerhof and Lohmann state that iodoacetate muscles in rigor contain the amount of triosephosphate calculated from the concentration of hexosediphosphate and the equilibrium constant of the system, as determined upon extracts. It was not stated how these muscles were prepared for analysis. Since there was a possibility that they had not been frozen, but merely crushed into trichloroacetic acid, a few experiments were performed in which this procedure was used instead of freezing the muscles. When this was done, triosephosphate was found, in amount of 5 to 7 mgm. per cent of P, values which correspond to the equilibrium conditions found by Meyerhof and Lohmann. It is improbable that any breakdown of hexosediphosphate to triosephosphate, or the reverse of this, takes place while the muscle is frozen, even if it be regarded as a supercooled liquid rather than a solid. If the triosephosphate had formed in the rigor muscles, and the equilibrium had shifted in the freezing process to that which obtains at 0°, there should have been 2 or 3 mgm. per cent of triosephosphate-P present. The absence of even this amount indicates that the substance is formed only when the cell membrane is injured. Under these circumstances, it is doubtful that the compound plays any part in the anaerobic metabolism of the muscle cell.

This finding indicates that the mechanism of lactic acid formation in the intact cell may be different from that found in the extracts. If the first point of attack of iodoacetic acid in extract is to inhibit a reaction which does not take place in the cell, then it is necessary to postulate either that the enzyme poison has a different action in extract than it has in the cell, or else that a different mechanism of lactic acid formation, also inhibited by iodoacetic acid, is being utilized by the cell than the usual one found in extract. Acceptance of the former possibility requires adoption of the view that results obtained on extract are not necessarily transferable to the cell. There is a rather close analogy available for the latter possibility: J. Needham and his co-workers (1937) have found that the chick embryo uses a non-phosphorylating mechanism of glucolysis, which is inhibited by fluoride, and cannot be demonstrated in extracts.

In this connection, the question arises of the significance of hexosemono-

phosphate formation. It has been postulated for normal muscle (Sacks and Sacks, 1933) that this reaction takes place directly from phosphocreatine and glycogen, rather than from inorganic phosphate, and that it is used to supply energy for anaerobic activity whenever the formation of lactic acid is inadequate. The same interpretation will fit the situation in the poisoned muscle. Other procedures which modify the course of anaerobic metabolism have given results which are in harmony with this interpretation. For example, Kerly and Ronzoni (1933) found that in resting anaerobiosis of frog muscle in a medium of pH 6.0, hexosemonophosphate was formed as long as any phosphocreatine was present; after this substance was exhausted, lactic acid formation set in, but the accumulated hexosemonophosphate did not disappear. In a medium of pH 9.0, lactic acid was the sole product formed. These data are difficult to interpret except on the basis that hexosemonophosphate formation and lactic acid formation are independent reactions which can substitute for each other as conditions demand.

Another point raised by the present data is whether the formation of hexosediphosphate can be considered a normal process. It takes place in muscle poisoned by iodoacetic acid, and then only after the onset of contracture, that is, in cells which have undergone an irreversible change. Other enzyme poisons, such as fluoride, also lead to the formation of this substance. In the absence of such agents, it is only in muscle which has been minced post-mortem and then incubated, that it is formed (Milroy, Beattie and Lyle, 1933). When a substance appears only under such abnormal conditions, there is as much likelihood that it is an artefact resulting from the operations to which the tissue has been subjected as that it is a normal metabolite.

It is seen from the data here presented that there is no necessary correlation between the reactions taking place in anaerobic contraction of muscle poisoned with iodoacetic acid and the reactions which can be demonstrated in cell-free muscle extract which has been similarly poisoned. Two reactions have been found in the muscle which have not been found in the extract: direct interaction of phosphocreatine and glycogen to form hexosemonophosphate, and the formation of hexosediphosphate from the monophosphate and inorganic phosphate. On the other hand, two reactions which are known to take place in the extract have not been found in the muscle itself: formation of hexosemonophosphate from glycogen and inorganic phosphate, and conversion of hexosediphosphate to triosephosphate. However, the reactions of the phosphorus compounds which take place in the poisoned muscle before the onset of contracture are the same ones which take place in normal muscle contracting anaerobically. Such findings indicate the necessity for caution in using extract studies to interpret mechanisms which are operating within the cell.

SUMMARY AND CONCLUSIONS

1. In anaerobic contraction of frog muscle poisoned with iodoacetic acid, the formation of hexosemonophosphate takes place directly from phosphocreatine and glycogen.

2. Formation of hexosediphosphate takes place only after the onset of irreversible changes in the muscle, and is therefore not necessarily to be regarded as a normal process.

3. The formation of hexosemonophosphate is a reaction independent of lactic acid formation, which serves as a substitute source of energy under anaerobic conditions when lactic acid formation is inadequate or inhibited.

4. Triosephosphate is not present in poisoned muscles in rigor which contain large amounts of hexosediphosphate.

5. The inhibition of lactic acid formation in iodoacetate poisoned muscle is due to some other effect of this substance than the inhibition of the oxido-reduction of triosephosphate.

6. The reactions of the phosphorus compounds which take place in anaerobic contraction of the poisoned muscle before the onset of contracture are the same ones found in normal muscle.

7. The reactions which take place in anaerobic contraction of poisoned muscle are not necessarily the same ones which are found in muscle extract under comparable conditions; from this it is concluded that interpretations derived from extract studies may not apply to the mechanisms actually used in anaerobic contraction.

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OBSERVATIONS ON THE INNERVATION OF THE CORONARY VESSELS OF THE DOG[1]

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It has been generally held in the past that the innervation of the coronary vessels of the mammal is peculiar with respect to the systemic blood vessels in that the parasympathetics, the vagi, were considered to be coronary vasoconstrictors and the sympathetic nerves, primarily coronary vasodilators. The evidence for this comes largely from work reported by Wiggers (1), Anrep and Segall (2), Hochrein (3, 4) and Rein (5). However, in the case of both the vagus and sympathetic nerves, contrary results have been reported. Coronary vasodilators in the vagus nerves have been reported to exist by Meyer (6), Martin (7), Drury and Smith (8), Greene (9), Danielopolu and Marcou (10), Hinrichsen and Ivy (11), Kountz et al. (12) and Klisiecki and Flek (13). The subject has been recently reviewed by Wiggers (14) and further details need not be given here. Recently, Greene (9) has presented evidence tending to show that some sympathetic vasodilator fibers are contained in the lower cervical portion of the vagus trunks. More recently Heymans (15) and his collaborators, Jourdan and Nowak (16), have claimed that the sympathetic fibers which are bundled in the vagus join the vagus within the skull and originate in the medulla. However, Kabat (17) has cast grave doubt upon the interpretation of Heymans and his collaborators.

It has been argued that the supposedly peculiar innervation of the coronary vessels serves to permit their needed dilatation when other systemic vessels are constricted as is the case in exercise. This argument is fallacious, since such adjustments of coronary flow actually occur through dynamic and humoral control and not because of the peculiarity of anatomical bundling of efferent vasomotor fibers.

The discrepancy between coronary innervation and innervation of systemic vessels has induced us to reinvestigate the subject. In order to evaluate accurately the active changes in coronary vessel caliber due to vasomotor nerves, it is necessary 1, to use a measure of coronary flow which

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gives a reliable index of total changes in coronary caliber, and 2, to eliminate other factors which may affect coronary flow, viz., changing driving force in the coronary arteries, changing extravascular forces acting on the vessels, and extrinsic humoral mechanisms. To fulfill these conditions we used a preparation consisting of a head and isolated heart with fibrillating ventricles, both perfused with defibrinated dogs' blood at constant pressure and temperature. The method for the isolated heart perfusion has been described in detail elsewhere (18). This was modified by also perfusing

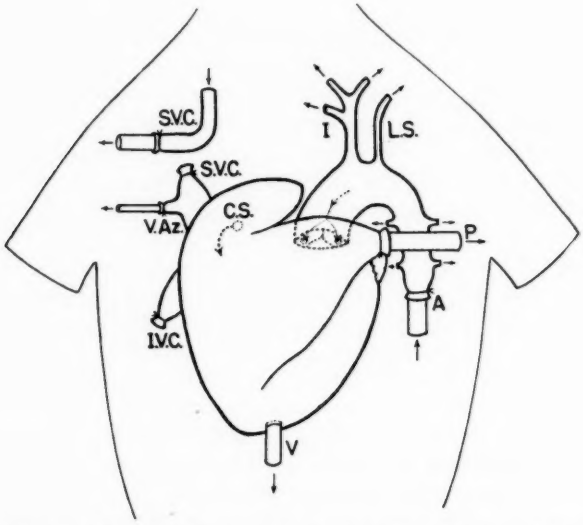

Fig. 1. Diagram of perfused head-heart preparation. *A*, cannula in aorta for perfusing head and heart; *I*, innominate artery; *L.S.*, left subclavian artery; *P*, cannula in pulmonary artery for collecting and measuring coronary flow; *V*, drainage cannula in left ventricle; *S.V.C.*, superior vena cava; *I.V.C.*, inferior vena cava; *V. Az.*, azygos vein; *C.S.*, mouth of coronary sinus. Blood from head and mediastinal structures is drained from *S.V.C.* and *V. Az.* back to aerator and perfusion apparatus. In this preparation the ventricles are fibrillating.

the head, neck and mediastinal structures from the same reservoir through the aorta to keep alive the brain and the cardiac nerves. The venous blood from these areas was drained from the superior vena cava and azygos vein and returned to the common reservoir (cf. fig. 1). With this preparation, coronary driving pressure and extravascular forces are kept constant, and extrinsic humoral effects are eliminated. As an index of coronary vessel caliber, total coronary flow from the pulmonary artery was measured.[2]

[2] We have found that an average of only 10 per cent of the total coronary flow

The direct effects of the vagi and sympathetic nerves were studied by stimulating the peripheral ends of the cut vagi and the stellate ganglia after double vagotomy. The tonic activity of the vagi and sympathetics was studied by observing the effects of vagotomy, spinal cord section above the level of the third cervical segment, and complete denervation of the heart, including the tying off of the blood supply to the head. Sodium barbital, nembutal, ether, or chloralosan were the several anesthetics used in these experiments. The type of anesthetic used had practically no effect on the nature of the results, although the last three anesthetics named yielded more reactive preparations than the sodium barbital.

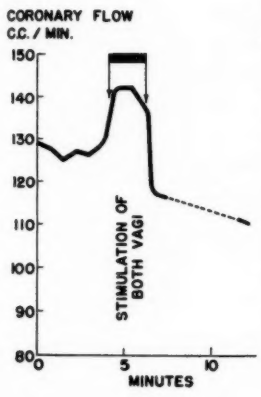

Fig. 2

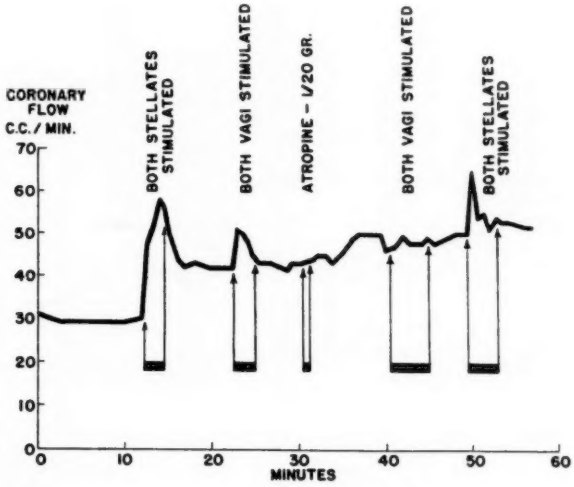

Fig. 3

Fig. 2. Coronary vasodilator effect of stimulating peripheral ends of cut vagi.

Fig. 3. Chart of coronary flow showing vasodilator effect of vagus stimulation and its abolition by atropine, and vasodilator effect of sympathetic stimulation and its persistence after atropine, demonstrating the cholinergic nature of former and adrenergic of latter.

I. Effect of stimulation of the peripheral end of the cut vagus. In 10 experiments it was found that stimulation of the peripheral ends of the cut vagi gave rise only to vasodilatation (cf. fig. 2). This dilatation was abolished or greatly reduced following the injection of 2.5 mgm. of atropine sulphate (cf. fig. 3). This indicates that the vagi contain cholinergic vasodilator fibers. In 25 experiments vagus stimulation gave no effect. No evidence was obtained at any time in the whole series of 35 experiments

drains through the thebesian vessels into the left ventricle (21). Thus the flow from the pulmonary artery represents practically the total coronary outflow.

that stimulation of the vagi within the range of current strength available with the Harvard inductorium gave coronary constriction.

II. Effect of stimulating the stellate ganglia with the vagi cut. In 7 experiments stimulation of the stellate ganglia gave rise to coronary dilatation, and this dilatation was not abolished by atropine even in doses sufficient to abolish the vagus dilatation (cf. fig. 3). This indicates that there are adrenergic coronary vasodilators in the sympathetic nerves. In 5 experiments coronary vasoconstriction was obtained following sympathetic stimulation (cf. fig. 4). In 27 experiments a biphasic or doubtful response was obtained. We have not been able to test the effect of ergotamine or

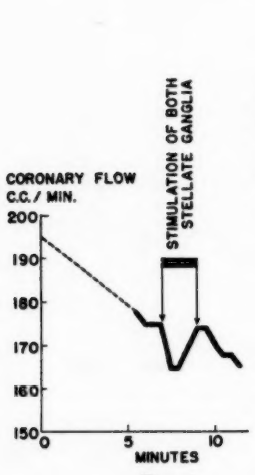

Fig. 4

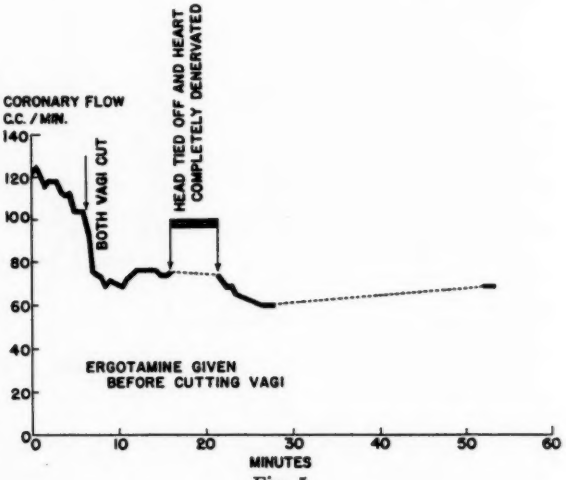

Fig. 5

Fig. 4. Coronary vasoconstrictor effect of sympathetic stimulation.

Fig. 5. Chart showing vasoconstrictor effect of cutting both vagi, and further vasoconstriction after completely denervating the heart. Experiment was done after 10 mgm. of ergotamine tartrate were given.

the dioxane derivative F 933 upon this coronary constriction. Nevertheless, our results suggest that the sympathetics contain adrenergic coronary vasoconstrictors.

III. Effect of cutting the vagi in the innervated isolated fibrillating heart preparation. In 8 experiments it was found that cutting the vagi led to a decrease in coronary flow. This occurred in 1 preparation in which ergotamine (10 mgm. of tartrate) was first given (cf. fig. 5), and it occurred in 1 experiment in which the sympathetic pathways had been severed by sectioning the spinal cord above the level of the third cervical segment prior to cutting the vagi (cf. fig. 6). These results, therefore, confirm

the fact that the vagi contain only coronary vasodilator fibers, and indicate that these are under the tonic influence of centers in the central nervous system. Our results confirm our findings with nerve stimulation that these dilator pathways are not adrenergic fibers of sympathetic origin which are contained within the vagus trunks but are truly cholinergic fibers arising from a parasympathetic center.

IV. Effect of sectioning the sympathetic pathways to the heart in the isolated fibrillating heart. Twenty-three experiments were done. In 14 cases the effect of sympathetic denervation was studied by noting the consequence of completing the denervation of the heart after the vagi had been cut. In

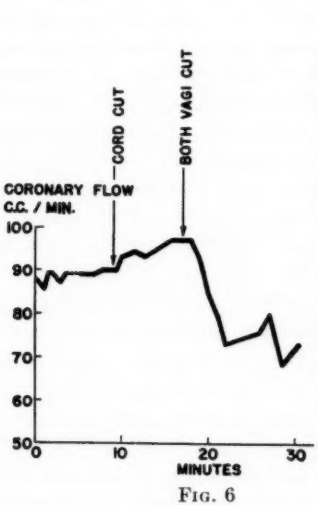

FIG. 6

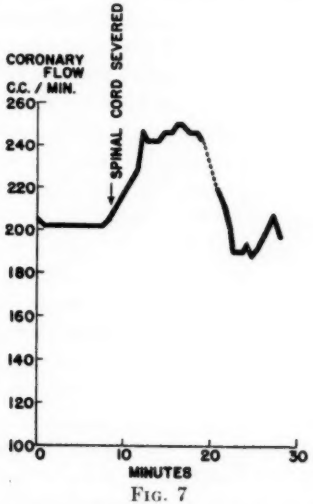

FIG. 7

Fig. 6. Chart showing vasodilator effect of severing spinal cord above C 3, and subsequent vasoconstriction after cutting both vagi.

Fig. 7. Vasodilator effect of spinal cord section above C 3 (both vagi previously cut).

9 cases the effect was studied by cutting the spinal cord above the level of the third cervical segment either before or after the vagi had been severed. In all instances where a change in coronary flow occurred, there was an increase (cf. fig. 7) confirming our previous results indicating the presence of sympathetic coronary vasoconstrictor fibers, and showing further that these fibers are under the tonic control of centers in the central nervous system.

In 2 experiments the denervation was performed after ergotamine (10 mgm. of tartrate) had been injected. In one of the experiments sympathetic denervation caused a decrease in coronary flow over and above that

previously noted in the same preparation on severing the vagi (cf. fig. 5). This finding would indicate that the tonic vasoconstrictor influence of the sympathetic is adrenergic in character and that there is a weaker adrenergic tonic coronary vasodilator influence distinct from the cholinergic dilator influence propagated by the vagi. This tonic adrenergic vasodilatation appears to be less powerful than the adrenergic vasoconstriction, since it is masked when ergotamine is not administered. However, faradic stimulation of the sympathetic fibers with a Harvard inductorium in our experience appears to act more readily on these adrenergic dilator fibers than on the adrenergic constrictor fibers.

V. Significance of results. Our results are in accord with our previous observations on the action of acetylcholine derivates and epinephrine (18) on this preparation of the dog, and appear to us to show conclusively that the innervation of the coronary vessels is similar to that of other systemic vessels as recently established, particularly by Burn (19). They show 1, that the vagi contain only cholinergic vasodilator fibers to the coronaries; 2, that the sympathetics contain both dilator and constrictor fibers of adrenergic type, and 3, that these fibers carry impulses from the central nervous system which exert a constant tonic effect regulating the caliber of the coronary vessels. Our results are not in accord with previous views on this subject and the discrepancy we believe is due to the different methods of study used. In previous work we believe the effects were masked by the action of these nerves on the aortic blood pressure, on the vigor of the contractions of the heart, on the secondary extracardiac humoral changes, on the development under certain circumstances of relative ischemia, and on the alterations in the distribution of coronary outflow between the coronary sinus and thebesian channels (cf. 20 and 21). Our method of study has avoided these pitfalls and has permitted evaluation of active changes in coronary caliber undisturbed by such extraneous factors.

The clinical importance of our work in relation to denervation of the human heart is apparent. If the coronary innervation in man is similar to that in the dog, then sympathetic denervation in man may not only interrupt afferent pain fibers but the only efferent vasoconstrictor fibers as well. This point of view has recently been put forth by Leriche and Fontaine (22) but without adequate evidence to support it. Our results supply the experimental evidence for this viewpoint.

SUMMARY

A method is described for determining the action of the sympathetic and parasympathetic nerves on the caliber of the coronary blood vessels in a preparation consisting of an isolated head and heart with fibrillating ventricles.

Evidence is presented showing that in the dog:

1. The vagi carry only cholinergic coronary vasodilator fibers which are tonically active. No evidence was obtained of cholinergic coronary vasoconstrictor fibers.

2. The stellate ganglia send to the heart adrenergic coronary dilator and adrenergic coronary constrictor fibers, both of which are tonically active.

3. The tonic action of the sympathetic nerves is predominantly vasoconstriction.

We are indebted to other members of the department for valuable aid in performing the experiments.

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THE EQUALITY OF THE CHLORIDE SPACE AND THE EXTRACELLULAR SPACE OF RAT LIVER

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Since the work of Urano (1908) evidence has been accumulating (reviewed by Fenn, 1936) which indicates the extracellular position of the chloride ion in muscle (see also Eggleton et al., (1937) Hastings and Eichelberger, (1937) and Gersh (1938)). The hypothesis has raised the question as to the confinement of chlorides to this phase in tissues other than muscle.

Manery and Hastings (1939) have recently noted that in a group of rabbit tissues including liver, sodium and chloride appear in serum ultrafiltrate proportions. This was considered to indicate that sodium and chloride belonged to the extracellular or connective tissue phase. Reasons were advanced for believing that these tissues contained a large proportion of cells which like muscle fibers were devoid of chloride.

The experiments reported here were undertaken in order to demonstrate by more direct means the position of the chloride ion in the liver. In histological sections of liver there are many areas which are not composed of liver cell cords. These areas represent a volume which will be termed extracellular in spite of the fact that they contain some cellular elements. We have compared this extracellular volume to the volume of the chloride space which is determined by assuming that all of the liver chloride is outside of hepatic cells and its concentration is the same as that in plasma (Maurer, 1938). The close agreement between the magnitudes of these two volumes led to the conclusion that they represented the same tissue phase. The cellular constituents, fat, glycogen and nitrogen, were varied in an attempt to alter the chloride space. In each case, however, the chloride concentration was found to vary directly with the corresponding histological extracellular volume and was independent of changes in the cellular constituents.

METHODS. White rats of the Wistar strain were used, weights being recorded at intervals for one to two weeks previous to the actual experiment as an index of apparent health. Five rats were used as normals

(group I); five received varying amounts of white phosphorus dissolved in almond oil and injected subcutaneously (group II); and four animals were placed on a high fat, low choline diet for 21 to 24 days (group III). This diet consisted of casein (24.5), cane sugar (46.2 per cent), salt mixture (5.3 per cent), and fat (24 per cent). The animals received no food or water for three hours before they were sacrificed. They were then stunned, the carotids cut, and the blood collected in a small beaker to which 2 mgm. of heparin had been added. The liver was exposed and 0.6–1.2 gram samples for glycogen analyses were cut from each of two lobes, blotted, and immersed in a weighed tube containing hot KOH. The remainder of the liver was then removed and weighed. Two pieces were removed for histological fixation and the rest, after the removal of hilus and large vessels was placed in a weighing bottle and minced. Aliquots were taken for analyses of chloride, potassium, water, nitrogen and blood content. Fat analyses were carried out on the samples used to determine water content. Chloride, potassium, and water were also determined on whole blood and plasma.

Chemical methods. Chloride was determined by the method of Van Slyke (1923–24) as described by Manery et al. (1938). Liver samples weighing between 0.5 and 1.0 gram and 0.2 cc. of whole blood or plasma were used for each determination. Duplicate analyses were done on plasma and liver with an average difference of 0.9 and 4.3 per cent respectively.

The method used for *potassium* analyses was based on the titrimetric procedure of Shohl and Bennett with the modifications reported by Fenn et al. (1938). Percentage recoveries of 103 per cent were obtained on K_2SO_4 solutions and 105–6 per cent on plasma-like solutions. The average per cent difference between duplicate analyses was 2 per cent with an occasional high value of 7 per cent.

To determine *water content* weighed amounts of liver were dried to constant weight at a temperature of 100° plus or minus 10°. The determinations were done in duplicate with an average difference of 0.16 per cent.

The *blood content* was estimated by the method used by Manery, Danielson, and Hastings (1938).

The *glycogen* method described by Good, Cramer and Somogyi (1933) was used with some of the modifications suggested by Nutter (1938). Aliquots of the hydrolysate were analysed by the Shaffer-Somogyi method (1933).

The *neutral fat* was estimated as described by Hastings and Eichelberger (1937), and the *protein* content determined by the macro-Kjeldahl method.

Histological method. The samples used for histological measurements were selected from the distal part of the liver in every case and as far as

possible from the same liver lobe in each animal. The samples were blotted to remove excess blood and were immediately placed in fixative solutions.

Tarkhan (1931) has described the shrinking or expanding of tissues caused by common histological reagents during fixation, dehydration, clearing, and imbedding. Hence the histological sections prepared probably are not exact reproductions of the fresh tissue. To determine whether or not the histological procedures produced differential changes in the tissue, two widely differing routines were used to obtain sections of each liver. No difference could be discerned between the two methods in the relation of liver cell area to the total area, indicating that any swelling or shrinking which occurred affected all parts of the liver equally.

The two procedures were as follows: 1. One sample was fixed for 48 hours in Bouin's solution, was trimmed into two cubes approximately 0.5 cm. on a side, which were then dehydrated with ethyl alcohol, cleared in xylol, and imbedded in paraffin. Sections were cut at three micra, and treated with Crossmon's (1937) modification of Mallory's connective tissue stain. 2. The second sample was fixed for 48 hours in a 15 per cent formalin solution, then washed overnight in running water, trimmed, and cut into three blocks not thicker than one millimeter, which were then placed in a Flemming's solution containing: 15 parts 1 per cent chromic acid (aqueous); 4 parts 2 per cent osmic acid; 1 part glacial acetic acid.

Forty-eight hours later the blocks were again placed in running water overnight. They were subsequently dehydrated and cleared with butyl alcohol and imbedded in paraffin. Again three micra sections were made and counterstained with Safranin O. The tendency for such thin sections to flake caused considerable difficulty until it was suggested that the cut face of the block be immersed in water for a period of 8 to 12 hours and transferred to ice water just prior to the actual sectioning.

The details of the histological method were worked out on cat liver. At first, camera lucida drawings were made of the histological section, but photomicrographs were later adopted as a more expeditious method. The photomicrographs ($3\frac{1}{4}$ by $4\frac{1}{4}$ inches; magnification 450 $\times$) were taken only of those areas in the slides which were free of central veins and interlobular masses of vessels, ducts, and connective tissue. Each photomicrograph was weighed and that part which was not composed of liver cells was carefully cut out with scissors. The paper representing the liver cells (fig. 1—cells only) was then weighed and its percentage weight ascertained. The remainder was that area which we wished to compare to the chloride space determined chemically.

The presence of interlobular vessels and ducts presented some difficulty since they were a part of the tissue used for chemical analyses but were not included in the part of the slide selected for photographing. From camera

lucida drawings of an entire section of each liver the area occupied by interlobular veins, arteries, ducts and connective tissue was estimated at 3 to 6 per cent. An average figure of 5 per cent was used for each animal. The sections used for these measurements being only 3 micra thick, the measured cell area can be assumed to represent accurately the relative cell volumes for that section without any danger of misinterpretation due to overhanging cell margins. If all sections of the liver are alike it also represents the relative cell volume of the whole liver.

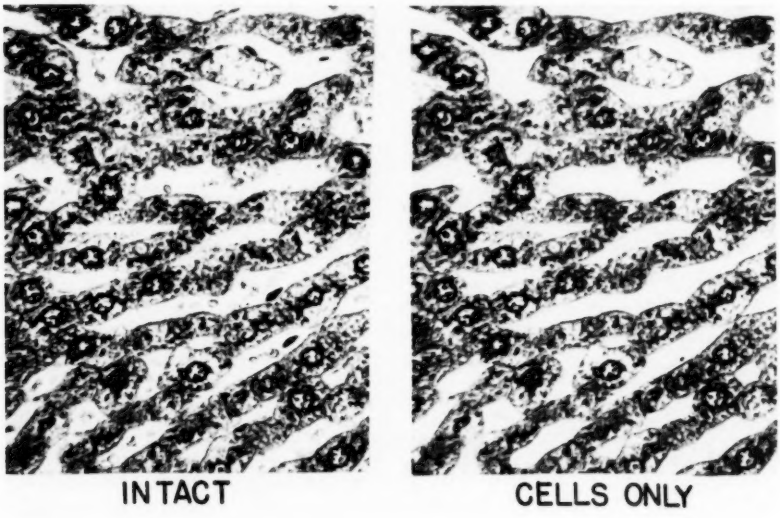

Fig. 1. Comparison between the intact photomicrograph (on the left) and the same (on the right) after cutting out the non-cellular parts with scissors. In this case the cells account for 77.3 per cent of the total area. Magnification 450 times.

Calculations. *Histological extracellular space.* For rat 1: area of liver cells in per cent of area photographed = 77.1 per cent. Area of liver cells in per cent of total liver = 77.1 × 0.95 = 73.2 per cent. Therefore the extracellular space = 26.8 per cent.

Ten photomicrographs were taken of every rat liver and the liver cell area was measured in each as indicated above. The average probable error in any series of ten was ± 2.0 (range ± 1.4 to ± 2.8).

Chloride space. This is calculated from the equation:

$$\left(\frac{\text{Total liver chloride} - \text{liver blood chloride}}{\text{plasma chloride}}\right) \times 0.96 \times 100 + \text{liver blood volume} = \text{chloride space}$$

For rat 1

$$\frac{28.1 - (.032 \times 85)}{104.6} \times 0.96 \times 100 + 3.2 = 26.4 \text{ per cent}$$

In this calculation 0.96 = r, the Gibbs-Donnan ratio, 85 = m.eq. Cl per liter of blood and 3.2 = cubic centimeter of blood per 100 grams of liver.

RESULTS. The histological determination of extracellular volume in the liver of a single cat gave a value of 29 per cent. This figure so closely approximated the average value obtained by Fenn (unpublished) for the

TABLE 1

RAT	LIVER							PLASMA Cl	Cl SPACE	
	Cl	K	H_2O	Glycogen	Nitrogen (× 6.25)	Fat	Blood		Chem.	Hist.
					Group I					
	m.eq./kgm.	*m.eq./kgm.*	*per cent*	*per cent*	*per cent*	*per cent*	*cc./kgm.*	*m.eq./l.*	*per cent*	*per cent*
1	28.1	94.8	70.1		21.3	0.8	32	104.6	26.4	26.8
3	26.3	93.0	68.4	4.1	22.2	2.0	24	101.6	26.3	25.6
4	24.6	96.1	69.5	3.4	21.9	1.9	24	104.8	23.1	23.4
13	25.8	95.5	68.1	6.2	20.6	0.5		107.8	23.5	24.0
14	26.6	98.4	68.8	3.3	23.6	0.8	24	100.5	26.0	25.4
					Group II					
6	25.4*	98.3	70.4	2.5	20.6	3.2*	18	104.6*	23.6	26.6
7	22.2	93.8	69.3	0.4*	18.5	10.1*	19	107.9	20.3	19.5
8	25.3	96.0	69.7	4.5	18.9	4.0	23	112.7	22.1	24.2
16	31.3	101.9	69.5	3.7	22.4	1.7*	41	110.3	28.1	25.3
17	32.6	94.7	69.4	3.1	22.4	2.6	30	110.5	29.0	26.0
					Group III					
9	29.4	102.5	67.8	3.1	22.1	4.7	46	111.9	26.7	23.5
10	29.7	101.9	67.9	1.0	23.2	5.0*	42	113.9	26.2	22.8
11	24.4	98.4	68.0	0.9	23.8	4.4	27	108.2	22.3	21.7
12	29.4	99.8	67.8	2.5	22.7	3.1	28	113.2	25.9	23.5

The figures for nitrogen and blood content represent single determination; all others except those with asterisk are averages of duplicates.

chloride space of the same tissue that the method was considered sufficiently quantitative for further investigation. It was therefore applied to a series of fourteen rat livers on which the chloride space was also determined chemically. The results from both types of determinations are shown in the last two columns of table 1. It is evident that the two methods are in essential agreement, the average value for the chemical Cl space being 25.0 per cent (p.e. ± 1.7) and for the histological extracellular space 24.2 per cent (p.e. ± 1.3).

Rats 1, 3, 4, 13, 14, were normal animals, and the data from these are outstanding not only because of the close equality found between the volumes of the chloride spaces and histological extracellular spaces, but also because the values obtained with the different animals were so constant.

Rats 6, 7, 8, 16, 17 were animals injected with white phosphorus. The first three were killed when clinical signs (anorexia, etc.) of phosphorus poisoning appeared. Only one of these (no. 7) showed any significant increase in fat content and decrease in glycogen and protein. Rat 8 is of interest in that its nitrogen was as low as that of rat 7, but its glycogen and fat were both high normal. Rats 16 and 17 were fed a high carbohydrate diet after poisoning had been accomplished. They had quite fully recovered when killed, as shown by their normal fat values and the additional fact that no fat was demonstrable histologically.

Rats 9, 10, 11, and 12 were fed a relatively low-choline high-fat diet (see methods). A slight general increase in liver neutral fat resulted. In rats 10 and 11 a decrease of glycogen and a corresponding increase in protein occurred.

Although the changes in the last 2 groups of animals were not as great as were anticipated, nevertheless, certain cellular constituents did vary in relation to each other. In spite of this the chemical chloride space always closely approximated the histological chloride space. The most reasonable interpretation of this fact is that chloride is confined to the histological extracellular volume, being in the same concentration there as in plasma, and that hepatic cells are devoid of chloride. If such were not the case and some of the chloride found by analysis belonged to the hepatic cells, a portion of the extracellular elements must necessarily contain very little chloride. Most of these elements, on the contrary, are known to have high chloride concentrations, e.g., plasma ultrafiltrate, connective tissues, and blood vessel walls (unpublished data); the others such as the Kupffer cells and the cells of the lining of the sinusoids and the bile ducts might be expected to be rich rather than poor in chloride.

If hepatic cells are normally free of and impermeable to the chloride ion, it must be supposed that the hepatic bile acquires its chloride directly from the extracellular space.

The changes in neutral fat were too small to be of special interest but the analyses seem to support the general finding that as glycogen increases neutral fat decreases. The glycogen, water and potassium values are more significant. Fenn (1939) has shown that glycogen is deposited in rat liver along with potassium and water in the same concentrations in which they accompany other liver solids. Our data are presented in confirmation of this finding. In figures 1 and 2 of Fenn's publication (1939) a slight decrease in water and potassium can be observed as glycogen increases from 0.2 to 4.0 per cent. If our data are added to these graphs the

decrease disappears and a horizontal straightline seems to be the best representation of the potassium-glycogen and water-glycogen relations. The average potassium content of whole blood was 51.1 m.eq. per liter and for plasma 7.5, both being relatively constant throughout the series of animals. Appropriate corrections have been made for blood and fat and for the potassium and water in the extracellular phase in order to estimate the amount of potassium and glycogen present per liter of cell water. When these are plotted the points again lie along a horizontal straight line showing that as the glycogen concentration per liter of cell water increases, the potassium is unchanged.

CONCLUSIONS

1. The extracellular volume (i.e., the volume of the liver not occupied by hepatic cells) as determined histologically is quantitatively equal to the chloride space.

2. This finding is presented in support of the view that hepatic cells are normally free of chloride and may be impermeable to it.

3. Confirmation is made of the work of Fenn and others which shows that glycogen is deposited in the liver along with potassium and water so that the concentration of these substances in the cell water remains unchanged.

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RESPIRATORY RESISTANCE, OIL-WATER SOLUBILITY, AND MENTAL EFFECTS OF ARGON, COMPARED WITH HELIUM AND NITROGEN[1]

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In a previous paper by Behnke and Yarbrough (1938) it was pointed out that the remarkable stupefaction and neuromuscular impairment experienced by deep sea divers at depths below 100 feet could be eliminated or minimized by substituting helium for atmospheric nitrogen[2] in the divers' air supply.

The reactions characteristic of air intoxication or narcosis at high pressures are essentially slowed mental activity, inability to perform efficent manual work, and emotional disturbances which may culminate in loss of consciousness at depths below 350 feet (Behnke, Thomson and Motley, 1935).

Oxygen as an etiologic factor in these disturbances was eliminated by the demonstration that pure oxygen could be breathed at 4 atmospheres' pressure equivalent to the partial pressure of oxygen in air at a pressure of 20 atmospheres (corresponding to a diving depth of 630 feet) for periods of time up to 15 or 20 minutes without symptoms (Behnke, Johnson, Poppen and Motley, 1935).

In continuing our investigations it seemed advisable to ascertain the rôle played by argon in producing these mental phenomena, comprising as it does about 1.2 per cent of the atmospheric nitrogen.

In consideration of the importance of this problem in deep sea diving, in the treatment of respiratory obstruction, and in the study of the manner of action of the inhalation anesthetics, we have measured or described the breathing resistance, the oil-water solubility ratio of argon, and the mental impairment induced by breathing argon compared with helium and nitrogen.

1. *Respiratory Resistance of Argon Compared with a Mixture of Helium and Oxygen, and with Air. Method of procedure and experimental data.*

[1] The subject material in this article should be construed only as the personal opinion of the writers and not as representing the opinion of the Navy Department officially.

[2] Includes argon and the rare gases.

Resistance to simulated vigorous respiration was tested by attaching a Benedict spirometer to a respiratory resistance machine[3] designed to determine resistance to breathing encountered in respiratory apparatus.

This appliance consists essentially of bellows controlled by a variable crank mechanism which could be operated at the rate of 15 inspiratory and expiratory cycles per minute. At this rate of speed approximately 32 liters of gas were admitted to the spirometer and the same quantity of gas withdrawn over a period of a minute. The resistance encountered in the to and fro passage of gas from bellows to spirometer was recorded by separate water manometers for each phase of the cycle. The smallest diameter in the circuit measured 0.75 inch at the junction of spirometer outlet and intake tube of resistance machine.

Successive measurements of resistance to gas flow were made with the spirometer filled with air, then with 80 per cent helium-20 per cent oxygen,

TABLE 1

Measurements of resistance to gas flow (cm. of water). Average of values for inspiration and expiration

PRESSURE	GAS TESTED		
	Air	80 per cent He 20 per cent O_2	86 per cent argon 14 per cent nitrogen
atms.			
1	3.25	2.15	3.65
2	4.60	2.85	5.05
3	6.10	4.05	6.95
4	7.50	4.95	8.20
Average.....	5.36	3.50	5.96

and finally with 80 per cent argon-14 per cent nitrogen. The results of these tests at pressures of 1 to 4 atmospheres are recorded in table 1.

Discussion of data. Under the conditions of our experiments the values for the machine tested resistance of the different gases were roughly proportional to the square roots of the gas mixture densities (specific gravities) if the density of air is taken as unity. As pointed out by Kernan and Barach (1937) Graham's law postulating that the rate of diffusion of a gas is inversely proportional to the square root of the density is applicable to the problem of breathing resistance of gases since it also governs the rate of effusion, or the passage of a gas through small orifices.

Barach (1936), in addition, found that the substitution of 80 per cent helium-20 per cent oxygen for air brought about a 30 to 50 per cent reduction in the pulmonary airway pressure of subjects breathing violently.

[3] Mine Safety Appliance Company, Pittsburgh.

Our results make an interesting comparison with Barach's data. The substitution of the helium-oxygen mixture for air decreased the pressure in the resistance machine 33 per cent, a value 9 per cent lower than the theoretical value calculated according to Graham's law. The argon mixture, on the other hand, increased the pressure 11 per cent compared with air, which is in agreement with the theoretical value.

Since the elevation of barometric pressure served to increase the density of the gas mixtures, at 4 atmospheres' pressure somewhat more than twice the resistance to gas flow developed compared with surface (1 atmosphere) values.

If Graham's law is also applicable at decreased barometric pressures, then the breathing of helium-oxygen at normal barometric pressure is equivalent insofar as respiratory resistance is concerned to breathing air at an altitude of about 18,000 feet (0.5 of an atmosphere).

With reference to actual respiration tests we could not detect any difference in resistance encountered in breathing helium-oxygen, air, or argon-oxygen at atmospheric pressure. At a pressure of 4 atmospheres there was also no appreciable subjective difference in respiratory resistance. The altered mental state, however, induced by atmospheric nitrogen when air was breathed, and by argon containing 20 per cent oxygen, was manifest at this pressure.

At a pressure of 10 atmospheres, equivalent to a diving depth of 300 feet, Lt. Comdr. Momsen, U. S. N., and one of the authors were able to breathe argon-oxygen for a few minutes only because of the increased resistance to breathing and the narcotic action of argon.

2. *Solubility of Argon in Olive Oil and in Water.* The solubility of argon in olive-oil was determined by bubbling an 86 per cent argon-14 per cent nitrogen mixture freed from water vapor, through the oil at a temperature of 38°C. for a period of one hour, and subsequently extracting the gases by repeated evacuation in a Van Slyke extraction chamber.

The solubility of the argon mixture in water was determined in a similar manner except that the gases were saturated with water vapor prior to passage through a tonometer tube.

Calculations were made from the equations formulated by Van Slyke and Stadie for reducing the pressure of a gas extracted in vacuo to standard conditions of temperature and pressure. A correction was made for the nitrogen present in the gas mixture.

In table 2 are listed the solubility values obtained compared with previously determined coefficients for helium and nitrogen (Behnke and Yarbrough, 1938). The solubility coefficient α, represents the cubic centimeters of gas (0°C., 760 mm.) dissolved per cubic centimeter of liquid.

3. *The Mental State Associated with Respiration in an Argon-Oxygen Atmosphere Compared with Air. Experimental procedure and results.* The

argon-oxygen mixture used in these tests contained 69 per cent argon, 11 per cent nitrogen, and 20 per cent oxygen.

At atmospheric pressure the argon-oxygen combination was breathed by experienced subjects through a mask attached to a spirometer. The results at this pressure were negative; the subjects were unable to detect any difference either in breathing resistance or in their mental state when argon, air, or helium were breathed successively.

For tests at increased pressures, divers dressed in regulation diving suits were placed in a 12-foot cylindrical steel tank containing about 8 feet of water. Increased depths were simulated by raising the air pressure above the water to any desired level. Observers on the outside of the tank maintained continual telephone communication with the divers who also could be observed through the glass ports in the walls of the chamber.

TABLE 2

Argon, helium, nitrogen solubility in water and in olive oil at 38°C., and the respective oil-water solubility ratios

WATER			OLIVE OIL		
α Argon	α Helium	α Nitrogen	α Argon	α Helium	α Nitrogen
0.0262	0.00872	0.01275	0.1395	0.0148	0.0667

Oil-water solubility ratios

ARGON	NITROGEN	HELIUM
5.32:1	5.24:1	1.7:1

The divers tested in these experiments represent carefully trained men of exceptional stability. Their experience in breathing air at pressures up to 10 atmospheres (300 feet diving depth) ranged from 5 to 20 years. As a result of this experience they have long been accustomed to the progressive stupefaction and neuromuscular impairment induced by air as the pressure is increased from 1 to 10 atmospheres. Moreover, the divers have become proficient in estimating their diving depth based on subjective reactions usually well within 50 feet of the actual depth.

Since the lives of the divers frequently depend upon their awareness to any change in the gas mixture breathed or in any unusual subjective reaction, they may be regarded with respect to the condition of their gaseous environment as sensitive and reliable indicators.

The tests were conducted in such manner that the divers did not know either their actual depth or the nature of the atmosphere breathed. They were asked to state their depth on the basis of previous experience in high

air-pressure environments. The results of these tests when argon was breathed are recorded in table 3.

These results indicate that argon induces greater stupefaction and neuromuscular impairment than air (nitrogen).

In addition one of the authors breathed argon under conditions similar to those prevailing for the divers, and experienced the usual altered mental state in a dive to a depth of 160 feet. The reactions, however, possibly as a result of subjective reinforcement, did not seem to differ greatly from those induced by breathing air at the same depth. Before the termination of the dive, the argon effect became strikingly apparent when, with the substitution of air for argon the mental fogginess cleared to such degree that the subject reported that he was being brought to the surface, unaware, of course, that a substitution of gases had been made in the atmosphere.

Discussion of results. Since argon comprises 0.94 per cent of the atmosphere, at a pressure of 10 atmospheres the partial pressure of argon would

TABLE 3

Estimates of diving depth made by divers breathing argon-oxygen based on their experience in breathing air at high pressures

DIVER	ACTUAL DEPTH	DEPTH FELT ACCORDING TO SUBJECTIVE REACTIONS
	feet	
1	130	200
2	90	150
3	130	200
4	120	250

correspond to that exerted by 10 per cent argon at atmospheric pressure. The failure of argon in a concentration of 69 per cent to affect experienced subjects at atmospheric pressure indicates that the small percentage of argon in air does not play a material rôle in nitrogen narcosis at high air pressures.

The argon effect, however, was apparent at pressure levels corresponding to depths from 90 to 300 feet. We can conclude that the depressant effect induced by breathing argon is greater than that experienced when air (nitrogen) is breathed. At the 300-foot depth the factor of increased respiratory resistance complicates the interpretation of subjective reactions, but at depths of 90 to 130 feet there can be no doubt that the mental responses are not greatly influenced by the respiratory resistance of argon. Of interest in this connection would be determinations of carbon dioxide elimination when argon, helium, and nitrogen are breathed under pressure.

These results are of interest in relation to the study of all gases inducing

narcosis. Helium, for example, elicits negligible mental aberrations compared with argon, while intermediate in its effect is the action of atmospheric nitrogen. To what properties of these gases may be attributed their different degrees of narcotic activity?

Argon like helium is chemically inert. There are no known compounds of these substances. Nitrogen, on the other hand, possesses valences of two and five and combines with many substances. Insofar as its narcotic activity is concerned it appears to be chemically inert since it can be almost completely recovered from the body when oxygen is breathed (Behnke, Thomson, and Shaw, 1935). Physical rather than chemical properties should therefore be considered in explanation of the action of these gases. In this respect they are comparable to lipoid soluble narcotics which are thought to act in a physical rather than a chemical manner.

Nitrogen and argon possess oil-water solubility ratios somewhat greater than the ratio for ether, and three times greater than the corresponding ratio for helium. This fact might account for the comparative freedom from untoward mental effects of individuals in a helium-oxygen atmosphere at high pressures. On the other hand, the greater depressant action of argon compared with nitrogen may indicate that the comparative solubility in oil of the two gases (argon being twice as soluble as nitrogen) enters into the problem. The foregoing facts are in accord with the Meyer-Overton law.

Another physical property that might influence the activity of the three gases is their molecular weight. As suggested by Lt. Comdr. Momsen, helium with a molecular weight of 4 induces the least disturbance, while the molecular weights of 28 and 40 for nitrogen and argon, respectively, indicate their relative difference in narcotic effect.

For the purpose of this paper it is sufficient merely to record that there are subjective differences induced by breathing argon or nitrogen compared with helium, and to enumerate the physical characteristics of oil-water solubility, and molecular weight as possible factors responsible for these differences.

SUMMARY

1. Simulated breathing resistance was tested in a respiratory resistance machine which admitted to and withdrew from a Benedict spirometer 32 liters of gas per minute.

2. The tested gas mixtures were: 86 per cent argon-14 per cent nitrogen, 76 per cent helium-4 per cent nitrogen-20 per cent oxygen, and air.

3. At pressures of 1 to 4 atmospheres the resistance to the passage of these gases to and from the spirometer varied as the square roots of their specific gravities (air = 1).

4. The oil-water solubility ratio for argon is 5.32 to 1 compared with a

value of 5.24 to 1 for nitrogen, and 1.7 to 1 for helium. Argon is twice as soluble, however, in water and in oil compared with nitrogen.

5. The narcotic effect of argon is greater than that of nitrogen at high pressures of 4 to 10 atmospheres, corresponding to depths of 100 to 300 feet.

6. At a pressure of 1 atmosphere no difference could be detected between argon, nitrogen, or helium with respect to respiratory resistance or psychologic effects.

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